Assessing the efficacy and welfare impact of euthanasia methods for broiler chickens

A Thesis Submitted to the

College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Animal and Poultry Science

University of Saskatchewan

Saskatoon

By

Bethany Baker

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Abstract

This research investigated the efficacy and welfare impact of various euthanasia methods for broilers (hatchery and on-farm) by evaluating their ability to induce instantaneous insensibility and reliably result in death with minimal pain and distress. One component compared three on-farm euthanasia methods; manual cervical dislocation (CD), a mechanical cervical dislocation device (KED), and a non-penetrating captive bolt device (Zephyr). The physical damage resulting from the methods was evaluated on cadavers in the first experiment. This was followed by a second experiment using live birds evaluating the time to insensibility and death with each method. Another experiment investigated water deprivation and its effect on the efficacy of on-farm euthanasia. The KED occasionally resulted in incomplete spinal cord severing and frequently produced complex bone fractures. The Zephyr induced insensibility the fastest (under 2s post-application), however it was not reliable at successfully killing birds. CD had the shortest time to death (TTD) with 100% success rate; but had a longer time to insensibility than the Zephyr. Water deprivation increased time to death, but had no effect on the onset of insensibility. The second component examined hatchery euthanasia investigating the best method to use CO_2 for neonate euthanasia. Four gradual induction (GI) flowrates and immersion (IM) into a pre-filled chamber at concentrations of 70, 80, 90 or 100% CO_2, were evaluated focusing on measures of distress, insensibility and death. Distress was observed with all treatments. Distress occurred at CO_2 concentrations of 0.4-1.1%, insensibility occurred at 11-18% CO_2 and death at 61-78% CO_2. Compared to GI, IM was more efficacious at producing rapid insensibility and death with a shorter duration of distress. For GI, increasing flowrate resulted in a linear decrease in duration of distress, latency to insensibility and death. Overall, IM into 100% CO_2 was most effective, with the shortest time to insensibility and death, and lowest frequency and duration of distress. Lower IM concentrations also resulted in rapid insensibility, but increased measures of distress and TTD. In conclusion, none of the methods tested were completely efficacious and without negative welfare impact, however the majority were successful at inducing insensibility and death.

Acknowledgements

I want to take the opportunity to express my gratitude towards everyone that helped, supported and guided me during this process. The work presented in this thesis would not have been possible without each of you. First and foremost, I would like to thank my supervisor, Dr. Karen Schwean-Lardner for the opportunity to undertake this endeavour and for all her support, guidance and encouragement during this process. Thank you for giving me the chance to flourish and grow as a scientist and a person and for instilling a passion for research, teaching and poultry in me. Thank you to my committee members, Dr. Hank Classen, Dr. Jennifer Brown and Dr. Susantha Gomis, for all the insights, suggestions and advice throughout this process. Your help has been invaluable in the completion of this research and thesis. Thank you, Dr. Fiona Buchanan, for chairing my graduate committee and for finding the time to attend my committee meetings. Thank you to Dr. Bernard Laarveld, for replacing Dr. Buchanan as my committee chair during her leave. I would also like to sincerely thank my external examiner, Dr. Karen Christensen.

A special thank you to all the farmers that participated in this study, Nick Langelaar and Langelaar Poultry Farm Ltd., Scott Langelaar and Piro Poultry Farms Ltd., Wally Sloboshan and P2 Barns, Joel Sloboshan and PV Poultry Ltd., Regan Sloboshan, Jelmer Wiersma and Wiersma Farm Ltd., Collin Keets and KMG Farms Inc., Justin Wright and Ron Patterson with Rolling Prairie Farm 2 Ltd. Thank you for welcoming me onto your farm and providing cull birds for the trial; without you the research would not have been possible. My thanks also go out to Tennille Knezacek, for making the on-farm data collection possible, giving me the opportunity to present at industry meetings and for always having a welcoming heart and helping hand. Thank you, Jenny Nicholds, for the role you played in my project and teaching me invaluable skills and knowledge in poultry pathology. A special thank you to Dorien Brosnihan, for driving Shorty, myself and lots of samples around Saskatchewan, for helping with data collection and for always bringing great conversation. Thank you, Trisha Pinton, for all your tireless help collecting data for my project, and for making the many hours spent on-farm, on the road, in the lab and PDS, and getting rabies vaccines, as enjoyable as they were productive. I would also like to thank the past and present staff at the U of S Poultry Centre, Jocelyn Fournier, Robert Gonda, Mark Meier, and Jason Marshall, who assisted during my barn trials. Thank you for your help feeding and

caring for my birds, your help with data collections and all the times you helped problem solve and come up with nifty solutions. Also, thank you to the staff at Prairie Diagnostics Services, for always patiently answering my questions and allowing us to work in the necropsy laboratory. Steven Laycock, I would like to thank you for your generosity and willingness to aid with the CO_2 euthanasia research. Thank you, Trevor Crowe, for your help, advice and readiness to double-check my calculations throughout the CO2 euthanasia research. I would also like to thank my fellow graduate student for taking time out of their busy schedules to help with data collections, for being there when I needed them and for bringing poultry-based joy into my life.

I would like to acknowledge my collaborators at the University of Guelph, Dr. Stephanie Torrey, Dr. Patricia Turner, Dr. Tina Widowski, and thank them for their help and contribution to the project and for all the knowledge they have shared. Furthermore, I would like to thank my funding agencies for providing the financial support that made this research possible; Chicken Farmers of Saskatchewan, Poultry Industry Council, Canadian Poultry Research Council, Egg Farmers of Canada, Hybrid Turkeys, the Ontario Ministry of Agriculture, Food and Rural Affairs, and Natural Sciences and Engineering Research Council.

I must give special thanks to all my friends and loved ones, who have supported me throughout the entirety of this process. Thank you, for keeping me sane, being there for me in the difficult moments, bringing joy into my life, and generally being amazing human beings. There are too many of you to name you all personally, but each one of you has played a role in getting me through to this stage, and I want to thank you for this. Even those of you that are not really sure what it is exactly I do up at the University but still fully support me anyway.

Speciale dank gaat uit naar mijn ouders. Mama en Papa, ik dank jullie voor jullie jarenlange inzet en dat jullie er altijd voor hebben gezorgd dat wij als kinderen alle mogelijkheiden en steun kregen de nodig was om onze talenten te ontwikkelen en te kunnen worden wie wij zijn. Zonder jullie onaflatende steun gedurende mijn studies en het schrijven zou ik dit niet bereikt hebben. Dankuwel, voor jullie geduld en bereidheid om er altijd voor mij te zijn ook al zijn jullie zo ver weg. Jullie stonden altijd klaar om mij te helpen als ik jullie nodig had, elke keer dat ik even mijn ei kwijt moest of een klankbord nodig had, alles wat jullie voor mij kritische gelezen en verbetert, en de vele keren dat jullie met een nieuw inzicht kwamen. Bedankt voor alles.

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List of Abbreviations

Ar	argon
C0	skull
C1	atlas (first cervical vertebrae)
C2	axis (second cervical vertebrae)
CD	manual cervical dislocation
CN	convulsions
CO_2	carbon dioxide
COM	cessation of movement
CRB	cessation of rhythmic breathing
CRD	completely randomized design
CSF	cerebral spinal fluid
CW	cloacal winking
EEG	electroencephalogram
FE	feather erection
GI	gradual induction
GS	gasping
HCO_3^-	bicarbonate ion
HS	head shaking
IM	immersion induction
KED	koechner euthanasia device
LOP	loss of posture
MCD	manual cervical dislocation
N_2	nitrogen
NPCD	non-penetrative captive bolts

O_2	oxygen
***p*CO_2**	partial pressure of carbon dioxide
***p*O_2**	partial pressure of oxygen
RCBD	randomized complete block design
SEP	somatosensory evoked potentials
TED	turkey euthanasia device
TTD	time to death

1.0 Chapter One - Literature Review

1.1 Introduction

More than 761 million broiler chicks were placed into production in Canada in 2017, resulting in over 715 million broilers being produced for human consumption (Statistics Canada, 2018). Globally, approximately 66 billion broilers were raised for consumption in 2017 (FAOSTAT, 2018). During poultry production, not all birds will progress from hatching to slaughter, and mortality rates range on average from approximately 1-6% in Canadian broiler flocks (Chicken Farmers of Canada, 2018). This translates to approximately 45 million placed broiler chicks not reaching slaughter for that year (Statistics Canada). Those birds either died or were culled on-farm. The euthanasia of birds during production is an important component of ensuring bird welfare; it is important for disease control purposes, as well as to end the suffering of birds that have no chance of recovery. To ensure bird welfare, euthanasia needs to be performed by techniques appropriate for broilers, thus research is needed to understand the various techniques and how they affect the bird's well-being.

In recent years, the scientific community has increased its understanding of sentience, affective states and the underlying biology of the well-being of animals, and these states are currently being used to evaluate the welfare of animal management practices (Miele et al., 2013). Relative sentience of birds compared to mammals has also changed in the public opinion, as families of species such as Corvidae show high levels of technical and social skills. Contemporaneously, the public eye and legislative bodies are becoming more concerned with the welfare of production animals and husbandry practices, with changes in legislation in relation to animal welfare occurring globally (Miele et al., 2013; Grethe, 2017). Consequently, research assessing the impact of standard husbandry or management practices on animal welfare has increased. One of these management practices related to broiler production that has come under public scrutiny in Canada in recent years is euthanasia, both on-farm euthanasia as well as the disposal of chicks.

Euthanasia is a manner of terminating animal suffering by ending the life of an animal when there is no reasonable chance of recovery in order to eliminate pain and distress (AVMA, 2013). The action of killing an animal is only euthanasia when it helps to end poor welfare of the animal, if the animal benefits from the ending of its life, and it is done with as little distress as

possible (Broom, 2011). Throughout the poultry production system, when an animal is suffering, timely euthanasia is beneficial to both the welfare of an individual animal that is suffering and the welfare of the flock as a whole. It also has an economic benefit to the producer by increasing overall flock health and thus productivity (Turner and Doonan, 2010). Although the task of euthanasia can be unpleasant, it is a more humane option than allowing a diseased animal to die of its own accord.

Euthanasia is an essential component of flock management that every poultry producer should utilize when necessary. Birds within a flock showing signs of illness or disease, an injury or that are clearly moribund should be promptly euthanized to minimize suffering of the animal in question, and to prevent the transfer of disease vectors to other birds within the flock. Birds that are unfit for transport and slaughter also require euthanasia to prevent suffering that could occur during the transport of such birds. In Canada, although there is legislation that protects farm animal welfare, there is no specific legislation in regards to the on-farm euthanasia of animals. However, Canadian poultry producers are expected to follow voluntary codes of practice, which contain requirements and/or recommendations on the best practices for euthanasia. The Codes of Practice for the Care and Handling of Hatching Eggs, Breeders, Chickens and Turkeys, covers broiler chickens, and states that competent personnel are required to recognize and treat or euthanize all sick or injured birds promptly. The document also gives requirements and/or recommendations and guidelines in regards to on-farm euthanasia methods and decision making (NFACC, 2016a). Producers that raise broilers under the *Raised by a Canadian Farmer Animal Care Program* of Chickens Farmers of Canada are required to adhere to specific animal care standards; this includes standards for euthanasia that may exceed the minimum requirements of the NFACC guidelines (Chicken Farmers of Canada, 2018). Furthermore, poultry production, in Saskatchewan falls under provincial legislation, specifically the *Animal Protection Act*, the *Animal Products Act* and the *Livestock Dealer Act*, which are in place to protect the welfare of animals. The *Animal Protection Act* protects all animals from distress, whilst the latter two acts protect farm animals during production, sale and transport (Canadian Food Inspection Agency, 2014). Although euthanasia is not specifically addressed in this legislation, a lack of the performance of timely euthanasia does fall under these laws. Furthermore, the federal *Health of Animals Regulations* requires that no sick, pregnant or unfit

animal is to be transported (Government of Canada Justice Laws Website, 2018a), hence preventing the transport of unwell birds necessitates their euthanasia prior to transport.

Within a hatchery setting, there is also a need for euthanasia. Newly-hatched chicks that are unviable, moribund or unlikely to survive and thrive during transport and placement should be euthanized to reduce the distress and possible suffering they would otherwise face. As with on-farm euthanasia, there is no specific legislation in regards to euthanasia in hatcheries, however they must adhere to the same legislation as those for poultry producers. Hatcheries are also are under specific additional legislation, the *Health of Animals Act* and the *Hatchery Exclusion Regulations,* which specify that hatcheries need to ensure that every chick shipped to farm is vigorous and healthy (Government of Canada Justice Laws Website, 2018b).

The importance of humane euthanasia to the welfare of animals and as a tool in humane poultry production means there is a need for scientific research to validate the effectiveness of various euthanasia methods with respect to bird welfare, and to aid in the decision making process in regards to humane poultry euthanasia. The need for increasing the scientific understanding surrounding euthanasia of broilers on-farm and in the hatchery has been identified as an animal welfare research need and a scientific research priority by the *Animal Welfare Research Needs for Chickens, Turkeys and Breeders (NFACC,* 2016b*), Poultry Code of Practice Scientific Committee for Chickens, Turkeys and Breeders (2013),* and the *Canadian Poultry Research Council's National Research Strategy (2012).* This research will ensure that the methods utilized for euthanasia are humane, and aid in encouraging producers to euthanize birds, rather than letting animals die of their own accord. With the increasing numbers of chicks being hatched and broilers produced globally, it is vital that humane euthanasia methods are available and timely killing is performed to promote the welfare of animals. The objective of this thesis is to provide scientific knowledge on humane euthanasia that will improve the welfare of broilers requiring euthanasia by increasing an understanding of the appropriateness of various euthanasia methods.

1.2 Euthanasia

1.2.1 What is euthanasia?

Euthanasia or "good death" is the action of ending the life of an animal in a manner that minimizes or eliminates pain or distress when the prospect of recovery is no longer likely (Erasmus et al, 2010 a,b,c; Gerritzen, 2013; AVMA, 2013; Thornber et al., 2014; Cors et al., 2015; NFACC, 2016a). For euthanasia to be successful it requires a rapid, irreversible loss of consciousness and then a prompt death via either respiratory or cardiac arrest or due to destruction of the brain function (Erasmus et al, 2010 a,c; Thornber et al., 2014; Cors et al., 2015; NFACC, 2016a).

There are three main mechanisms via which euthanasia methods can cause death, including hypoxia, direct depression of cortical neural system and physical disruption of brain activity or destruction of the brain (AVMA, 2013). Hypoxia will result in a depression of the neural system, and eventually loss of consciousness and death, due to a lack of oxygen (O_2) (ESFA, 2004). The lack of O_2 can be due to a displacement of O_2 in the respired air, as occurs with gaseous euthanasia, or due to reduced O_2 in the blood, such as with exsanguination (AVMA, 2002, 2013). The euthanasia methods that work via direct depression of cortical neural systems are agents that directly inhibit neurons in the brain that are involved in consciousness and sustaining life. Neuron depression causes a loss of consciousness, and the depression of the respiratory system results in cardiac arrest and death (AVMA, 2002, 2013). The final mechanism of euthanasia is physical disruption of neurons. Physical disruption can be done either via direct destruction of the brain by via concussive captive bolt stunning, blunt force or pithing, or via the inhibiting of neurons by electrical depolarisation of these neurons (AVMA, 2002, 2013). As the neurons involved in controlling cardiovascular and respiratory system are destructed, their activity will cease, resulting in death (AVMA, 2002, 2013).

1.2.2 On-farm euthanasia

Euthanasia is a vital part of good management and welfare for poultry farmers. When considering on-farm euthanasia, it is important that it is conducted in a timely manner, by a trained individual and with an appropriate euthanasia method. For the decision on the most appropriate euthanasia method to use, it is important to consider the methodology's ability to induce loss of consciousness and death with minimal pain and distress, its reliability and

irreversibility, as well as its safety for use and the emotional effect on the operator or personnel performing the procedure (Erasmus et al., 2010a,c; Thornber et al., 2014; Cors et al., 2015; NFACC, 2016a).

1.3 Consciousness and death

1.3.1 Consciousness and insensibility

Consciousness and the lack thereof play a vital role in assessing the quality of a euthanasia method. Although the exact definitions of consciousness differ from discipline to discipline, within poultry science and the scope of this paper, consciousness is an awakened state in which the bird is able to perceive, interpret, integrate and respond to stimuli both within the environment, with others and in itself (Hemsworth et al., 2009; Verhoeven et al., 2015). This means that an animal is able to experience unpleasant sensations such as pain or distress. The state in which there is a lack of consciousness is known as unconsciousness or insensibility. In this state of unawareness, there is a disruption to brain function, rendering the brain unable to receive normal stimuli and process sensory information (EFSA, 2004; Erasmus et al., 2010b; Terlouw et al., 2016a). In an unconscious state a bird no longer has the ability to perceive and experience sensations and emotions such as pain, suffering and distress (Adam and Sheridan, 2008; Hemsworth et al., 2009; Von Holleben et al., 2010; Erasmus et al., 2010b; Benson et al., 2012a,b; Verhoeven et al., 2015; Terlouw et al., 2016a). In order to minimize the possibility of the bird experiencing these, it is vital for any euthanasia method to result in a rapid insensibility (Benson et al., 2012a,b).

Within the avian brain there are three regions, the brain stem, thalamus and the pallium, that are integral in maintaining a conscious state (Reiner et al., 2005; Butler and Cotterill, 2006; Adam and Sheridan, 2008; Von Holleben et al., 2010; Erasmus et al., 2010b; Martin, 2015; Verhoeven et al., 2015; Terlouw et al., 2016a). The first region is the brain stem, and it contains an interconnected nuclei called the reticular formation. The reticular formation contains projection neurons that project to the thalamus, hypothalamus and pallium (Erasmus et al., 2010b; Terlouw et al., 2016a). The reticular formation maintains basic awareness, consciousness and level of arousal, as well as controlling consciousness of sensations by regulating which peripheral sensory signals get transmitted to the cortex (Adam and Sheridan, 2008; Erasmus et al., 2010b; Verhoeven et al., 2015; Terlouw et al., 2016a). In addition to consciousness, the

reticular formation also controls cranial nerves within the brainstem and nerves within the spinal cord, and via these controls, regulates movement, muscle tone, vital reflexes and autonomic functions (Erasmus et al., 2010b; Verhoeven et al., 2015). The thalamus and pallium perceive and integrate sensory inputs. Within these regions, a sensory stimulus, either from the environment or the bird itself, will be translated into a conscious sensory experience (Butler and Cotterill, 2006; Adam and Sheridan, 2008; Terlouw, 2016a).

Active functioning consciousness of the avian brain involves the pallium and thalamus for conscious perception and subjective experience, and the reticular formation of the brainstem to regulate consciousness (Verhoeven et al., 2015). Unconsciousness or insensibility occurs when there is a malfunctioning or disruption of activity in either the reticular formation within the brain stem, the neural pathways from the brainstem to the pallium, or within the cortex (Seth et al., 2005; Adam and Sheridan, 2008; Erasmus et al., 2010b). The disruption or damage of the brainstem or thalamus will abolish consciousness and inhibit activity within the pallium, while direct damage to the cortex will inhibit the sensations and perceptions involved in consciousness (Seth et al., 2005; Adam and Sheridan, 2008). The disruption of brain activity can be either due to direct injury to the specific brain regions, hypoxia, the depolarisation or hyperpolarisation of the neurons, or neurologic dysfunction resulting from concussion (Erasmus et al., 2010b; Terlouw et al., 2016a). As the unconscious state inhibits brain activity and the bird's ability to perceive and experience sensory stimuli such as pain and distress, it is essential that a method of euthanasia is able to rapidly induce a loss of consciousness, thus minimising the bird's exposure to negative experiences or affective states.

1.3.2 Death

Death is by definition the ending of the life of the animal as a whole, but can be described as the state of the animal once the brainstem ceases to function, combined with the cessation of vital functions of the respiratory and cardiovascular systems (Adam and Sheridan, 2008; Von Holleben et al., 2010; Martin, 2015). The total cessation of life is a result of the cessation of all the life functioning processes and organs within the animal. The processes vital for life are all dependent on one another for effective functioning and irreversible loss of one of these processes will result in the cessation of all the processes and eventually life (Adam and Sheridan, 2008; Martin, 2015). This is often classified as brain death, or respiratory or cardiac arrest (Thornber et

al., 2014; Cors et al., 2015; Martin, 2015). The brainstem is involved in the functioning of both the respiratory and circulatory systems, with the brainstem having direct control of respiration. Impairment to the brainstem or brain death will result in an abolishment of cerebral activity and consciousness, as well as a cessation of respiration, as the respiratory centres in the brainstem fail (Adam and Sheridan, 2008; Von Holleben et al., 2010; Sandercock et al., 2014; Martin, 2015; Terlouw et al., 2016a). Although brainstem death does not directly result in cardiac death, as the heart is autonomous and is able to function without the brain if it has a sufficient O_2 and energy supply and metabolic waste removal, the cessation of respiratory activity due to brain death will result in cardiac arrest (Adam and Sheridan, 2008; Von Holleben et al., 2010). When respiration ceases, this disrupts the supply of O_2 to the heart as well as the removal of waste from cardiac cells, inhibiting the proper functioning of the heart and resulting in cardiac death (Adam and Sheridan, 2008; Von Holleben et al., 2010). Cardiac death will also result in brain death, as the circulatory system is no longer able to supply oxygenated blood and energy needed for the proper function of neurons. As neurons cease to function, brain dysfunction will occur, causing a loss of consciousness and brain death (Adam and Sheridan, 2008). Euthanasia methods often aim at rendering the brainstem non-functional and inducing brain death, as this will result in a loss of consciousness and failure of the respiratory control centres, thereby ensuring cessation of respiratory activity followed by cardiac death. Once functioning of the brain and respiratory system has ceased, the cessation of the cardiovascular and all other process and organs within the organism will follow.

1.3.3 Indicators of insensibility and death

Consciousness, unconsciousness and death are difficult to measure directly and are often assessed by measuring other clinical signs. These include, reflexes or behaviours which are involved in brain function and aid in indicating the current state of the animal (Savenije et al., 2002; Verhoeven et al., 2015). As unconsciousness is due to the dysfunction of the brain, clinical signs that are associated with consciousness or insensibility can be tested and used to indicate whether the animal is unconscious. Similarly, as death is the result of a cessation of respiration, cardiac and brain death, clinical signals internally coordinated with these can be used as indicators of death (Savenije et al., 2002; Terlouw et al., 2016b). Reflexes are automatic movements resulting directly from a stimulus of the central nervous system and can indicate functioning of the brain stem and spinal cord (Verhoeven et al., 2015). When using reflexes or

behaviours as indicators, depending on the indicator itself, either its absence or presence can be indicative of unconsciousness or death. Involuntary behaviours and reflexive indicators of consciousness, insensibility and death have been validated and widely used in research in both on-farm, slaughterhouse and in laboratory settings (Gerritzen et al., 2004; Erasmus et al., 2010a,b,c; Sandercock et al., 2014; Martin, 2015; Woolcott, 2017; Woolcott et al., 2018a).

Indicators used to assess brain function and consciousness are separated into three types; brain stem or cranial nerve reflexes, spinal reflexes and behavioural reflexes. Cranial reflexes or brainstem reflexes are indicators of the brain stem functioning, as they are reflexes based on the cranial nerves which originate from the brainstem without spinal cord involvement and are not controlled by the pallium and cortex (Erasmus et al., 2010b; Von Holleben et al., 2010; Verhoeven et al., 2015; Terlouw et al., 2016b). Thus the absence of cranial nerve or brainstem reflexes is indicative of an impairment or dysfunction of brain stem activity and indicates that the animal is unconscious (Von Holleben et al., 2010; Verhoeven et al., 2015). Although the absence of these reflexes is indicative of unconsciousness, the presence of these reflexes is not conclusive evidence that the animal is conscious (Verhoeven et al., 2015). Corneal or blink reflex, pupillary dilation and nictitating membrane reflex are three common validated brainstem reflexes used to indicate unconsciousness. For the palpebral blink reflex or corneal reflex, sensory information, such as touch of the cornea or eyelid, is transmitted to the brainstem via the trigeminal nerve (cranial nerve V), and this sensory input will elicit a motor response by the facial nerves (cranial nerve VII) to close the eyelid, resulting in a blink (Erasmus et al., 2010b; Martin et al., 2016; Terlouw et al., 2016b). In response to a touch of the cornea or eyelid, the trigeminal nerve (cranial nerve V), also results in a reflexive closing of the nictitating membrane. The activation of the brainstem via the trigeminal nerve will elicit a motor response by the abducent nerve (cranial nerve VI), causing the nictitating membrane to close horizontally across the eye (Stibbe, 1928; Martin et al., 2016; Terlouw et al., 2016b). In response to light exposure, the pupil reflexively dilates, as the optic nerve (cranial nerve II) relays the sensory information to the brainstem, eliciting the subsequent pupillary response via the oculomotor nerve (cranial nerve III). The absence of a response by the pupils, or the mydyrasis indicates the brainstem has been rendered non-functional. The neural circuitry underlying these reflexes is also responsible for consciousness, thus an absence of these brain stem reflex indicators indicates the brainstem is non-functional, and insensibility or brain death has occurred.

Spinal reflexes are indicative of spinal cord activation, which does not directly translate to an animal's conscious state. However, flexor or polysynaptic reflexes, which involve activation of interneurons within the brainstem or higher regions of the brain, can be used to assess unconsciousness (Verhoeven et al., 2015; Terlouw et al., 2016b). Spinal reflexes involving cortical input can be used as indicators of consciousness include righting reflex and the pain withdrawal reflex, such as the pedal reflex or comb pinch (Verhoeven et al., 2015; Terlouw et al., 2016b). Behaviours, which are controlled by the cerebral cortex or brainstem can be used as an indicator of a conscious state or death, commonly noted by their absence. When indicators are being used to assess unconsciousness it is important to qualify both an absence of indicators of consciousness and a presence of indicators of unconsciousness.

Indicators of death are those that indicate the absence of processes vital for life. These are indicators that confirm that brain death, or respiratory or cardiac arrest has occurred. The absence of brain activity, respiration and a heartbeat, which can be detected using tools such as an electroencephalogram (EEG) or an electrocardiogram, are the most definitive indicators of death, but these are not easily deployable on farm (Erasmus et al., 2010b; Terlouw et al., 2016b). Thus, involuntary behaviours and reflexive indicators are more commonly used to measure death. Rhythmic breathing is an involuntary behaviour, and the absence of rhythmic breathing can indicate brain dysfunction and respiratory arrest. The respiratory centre, which has neurological control of autonomic respiration, is housed in the brainstem, with nerves, including the vagus nerve (cranial nerve X), that control the respiratory muscles and subsequent inspiration and expiration. By alternately activating the muscles involved in inspiration and those involved in expiration, the respiratory centre in the brain regulates breathing and ensure it occurs rhythmically (Martin et al., 2016; Terlouw et al., 2016b). The cessation of rhythmic breathing indicates a lack of neurological control over respiration due to an impairment of the respiratory centre and dysfunction of the brainstem. As the brainstem is vital to consciousness, the impairment to neural circuitry for rhythmic breathing also indicates insensibility or brain death (Martin et al., 2016; Terlouw et al., 2016b). The absence of breathing, rhythmic or otherwise, will result in anoxia, and subsequent brain and cardiac dysfunction (Von Holleben et al., 2010; Terlouw et al., 2016b).

The occurrence and cessation of convulsions, which are involuntary and uncontrolled neuromuscular spasms, are also widely used in research as an indicator of death. Convulsions are usually biphasic; the first phase is clonic convulsions followed a period of tonic convulsions. With clonic convulsions, there will be a spastic movement of the wings and legs, often classified as wing-flapping and leg paddling. Tonic convulsions involve muscle rigidity in the legs and outstretched wings, followed by some gentle leg paddling motion (Erasmus et al., 2010a,b; Dawson et al., 2007; Dawson et al., 2009; Woolcott, 2017). A sudden termination of brain activity results in the onset of convulsions (Dawson et al., 2007; Dawson et al., 2009; Erasmus et al., 2010a,b), as the absence of brainstem activity means there is a lack of modulation of the pallium, which alters nervous stimulation, causing uncontrolled muscular excitation and contractions (Martin et al., 2016; Verhoeven et al., 2016). Convulsions are incompatible with consciousness, so the onset and occurrence of clonic convulsions are indicative of insensibility (Dawson et al., 2007; Dawson et al., 2009; Verhoeven et al., 2015; Verhoeven et al., 2016). At the same time, the cessation of tonic convulsions has been validated as indicating that brain death and cardiac arrest have occurred (Dawson et al., 2007; Dawson et al., 2009; Erasmus et al., 2010a,b; Martin, 2015).

Cloacal movement or winking, the sporadic opening and closing of the cloacal sphincter, is controlled by the vagus nerve (cranial nerve X) and brainstem, and another indicator of brain death (Martin, 2015; Martin et al., 2016). The onset and cessation of cloacal winking is a conservative indicator of death, as it is the last indicator to occur (Martin, 2015; Martin et al., 2016). Feather erection, when the erection of feathers is sudden and global in an unconscious bird, occurs during the tonic phase of convulsions and is indicative of hypoperfusion or cardiac arrest (Heard, 2000; Erasmus et al., 2010b). The erection of feathers in this case indicates an end of the cardiovascular activity and brain death, as the dysfunction of the cardiovascular regulatory centre within the brainstem occurs due to brain death (Casey-Trott et al., 2013; 2014). Indicators of death start with the cessation of rhythmic breathing, followed by the occurrence of feather erection, the cessation of convulsions and finally cloacal winking followed by total muscle relaxation.

1.4 Euthanasia methods

1.4.1 Blunt force trauma and captive bolt

Blunt force trauma and captive bolt devices are two commonly utilized on-farm physical euthanasia methods that result in insensibility and death via the physical disruption of brain function (Galvin et al., 2005; Erasmus et al., 2010c; AVMA, 2013; Sparrey et al., 2014). Captive bolt devices can be split into penetrating and non-penetrating, however for poultry, only non-penetrating devices are commercially available (Martin, 2015). The mechanism of both non-penetrating captive bolt devices (NPCD) and blunt force trauma is concussive force, with the force of impact disrupting the neuron function throughout the brain, inhibiting the processing of sensory information and ability to maintain consciousness, and preventing O_2 flow to the brain eventually resulting in death (Galvin et al., 2005; Erasmus et al., 2010c; Bader et al., 2014; Casey-Trott et al., 2014; Sparrey et al, 2014; Cors et al., 2015; Martin et al., 2016; Terlouw et al., 2016a). With blunt force trauma, the percussive force is brought about by a sharp blow to the head by a solid object (Sparrey et al., 2014; Casey-Trott et al., 2014), whilst for the NPCD the percussive force comes from the bolt impacting the head. Whether from the object or the bolt, kinetic energy is released as it strikes the cranium and will have concussive effects resulting in traumatic brain injury and impairing brain structure and function (Martin, 2015; Woolcott, 2017). The initial impact causes coup damage of blood vessels and hemorrhage, contusions, axonal shearing and skull fracture, localized around the site of impact (Xiong et al., 2013; Martin, 2015; Woolcott et al., 2018a). The force from the bolt impact will cause a movement of the brain within the cranial vault and collide with the side of the cranium. This will cause contre-coup damage as the neurons and blood vessels stretch, tear, compress and deform, with the subsequent formation of contusion, hemorrhage and axonal shearing damage throughout the brain tissues and subdural space (Shaw, 2002; Gaetz, 2004; Andriessen et al., 2010; Martin, 2015; Grist et al., 2017). The initial damage resulting from the impact of the bolt and collision of the brain with the side of the cranium will initiate a series of metabolic, cellular and molecular events like cerebral oedema, hypoxia and ischemia, increased intracranial pressure, reduced blood flow to the brain, and glutamate neurotoxicity, causing further neuron death, tissue damage and atrophy (Gaetz, 2004; Pearce, 2007; Andriessen et al., 2010; Xiong et al., 2013). Hemorrhaging disrupts blood flow to the brain, altering intracranial pressure and causing cerebral ischemia. The contusions will also bring about oedema and ischemia, further increasing intracranial pressure (Martin, 2015; Woolcott, 2017). The concussion and traumatic injury

resulting from the initial bolt impact interrupts neuron function within the brainstem and pallium, inducing insensibility (Pearce, 2007). If the initial traumatic injury is severe enough, the cascade of metabolic, cellular and molecular events that occur in response to this damage will result in death.

Research has evaluated two commercially available NPCD for use in poultry. One study evaluated the use of the TED for 20kg turkeys and 3.5kg chickens by measuring the latency to cessation of the nictitating membrane reflex, respiration and heartbeat, and found a 100% death rate with insensibility occurring within 2 seconds and death within 2 minutes (Hulet et al., 2013). However, from this abstract, no information is available on sample size, other measurements used, or the results of this study. A more in-depth study by Woolcott and colleagues (2018a), investigated the effects of the TED and Zephyr-EXL on turkeys ranging from 4 to 20 weeks of age (1.3-14.9kg). This study established that an immediate loss of insensibility (no pupillary light reflex or nictitating membrane reflex present) occurred with 98% of Zephyr applications and 89% of TED applications. Time to death, as determined by latency to cessation of movement and cardiac arrest, was under 3 minutes for both devices. Neither device had a 100% success rate with first application. The study also found that both devices consistently caused significant physical trauma to the life-sustaining brain regions to disrupt brain function (Woolcott et al., 2018a). Research conducted by Erasmus et al. (2010a,c) on the use of the Zephyr with turkeys demonstrated similar findings with high hemorrhage scores and severe skull fractures indicating that the physical trauma resulting from the Zephyr was sufficient to directly disrupt brain function (Erasmus et al., 2010a,c). The study also found that insensibility occurred almost instantaneously, with the nictitating membrane reflex lost within 1 second of Zephyr application, and death occurring within 4 minutes (Erasmus et al., 2010a). A lower success rate was found for the Zephyr in this study compared to the rate reported by Woolcott et al., with 83% of attempts being successful at first applications (Erasmus et al., 2010a). These studies indicate that NPCD is successful in causing traumatic injury, disrupting brain function and rapidly inducing insensibility and death. Further research is needed to assess the suitability of NPCD, particularly the Zephyr, for on-farm euthanasia of broilers throughout the stages of production.

1.4.2 Cervical dislocation

Cervical dislocation is another technique available for use on the farm, in which insensibility and death are induced by separating the brain from the spinal cord and rupturing the carotid arteries (AVMA, 2013; Sparrey et al., 2014; Martin et al., 2016) via a luxation of the skull and cervical vertebrae. The severing of the spinal cord from the brain will inhibit communication between the brain and body, as the spinal cord is the main pathway via which information is transmitted between the two regions (Gregory and Wotton, 1990; AVMA, 2013; Bader et al., 2014; Sparrey et al., 2014; Martin, 2015; Martin et al., 2016). The severing of the spinal cord will result in injury to the spinal tissues, resulting in downstream damage via neurogenic shock, hemorrhage and apoptosis that will render the spinal neurons non-functional, inhibiting the transport of sensory information (Martin, 2015). The rupturing of the carotid arteries disrupts blood supply to the brain and alters arterial pressure leading to cerebral ischemia and hypoxia, rendering the brain non-functional, leading to insensibility and death (Gregory and Wotton, 1990; Bader et al., 2014; Sparrey et al., 2014; Martin, 2015). The reduction in supply of oxygenated blood to the brain from the artery rupture means there is insufficient blood provided to meet the metabolic demands of the brain resulting in cerebral ischemia, and the consequential reduction in O_2 supply means a lack of O_2 needed for the high metabolic requirements of the brain and spinal tissues causing hypoxia to occur (Martin, 2015a). The combination of insufficient blood and O_2 availability will result in neuronal dysfunction and eventually cell death, while the low blood pressure results in neurogenic shock (Martin et al., 2016). Cervical dislocation can be subdivided into manual or mechanical cervical dislocation (i.e. when a dislocation is performed with assistance of a specialized tool).

1.4.2.1 Manual cervical dislocation

With manual cervical dislocation (CD) the action of cervical dislocation is performed by hand and without the assistance of a tool, via a stretching and twisting of the neck. The bird is held in an upside down position, with one hand holding the bird's legs and the other holding the head. A downwards pulling movement followed by tipping of the head out and upwards is then applied and will result in stretching and twisting of the neck (AVMA, 2013; Martin, 2015). The stretching of the neck results in a tearing of the neck musculature, connective tissues and the blood vessels, followed by twisting or tipping of the head that will dislocate the cervical vertebrae and rupture the carotid arteries (Sparrey et al., 2014; Martin, 2015). Furthermore the

procedure results in extensive trauma to the brain stem and spinal cord as a result of the stretch force on the neurons and neural tissues, as well as resulting in concussion (Gregory and Wotton, 1990; Sparrey et al., 2014; Martin et al., 2016). The sudden trauma to the spinal tissue results in a wave of ascending electrical neural input overwhelming the brain, impairing its ability to function (Sparrey et al., 2014), which, combined with the effect of concussion on the brainstem, induces insensibility (Gregory and Wotton, 1990; Sparrey et al., 2014; Martin et al., 2016). To increase the likelihood of concussion occurring, the spinal cord should be severed close to or at the level of the brainstem, between the skull (C0) and the first vertebrae, altas (C1), or between the atlas (C1) and the second vertebrae, axis (C2) being ideal. As dislocations further down the spinal cord may not result in unconsciousness and lengthen the time to onset of insensibility (Gregory and Wotton, 1990; Sparrey et al., 2014). For young or very small poultry, a sharp edge has been suggested can be used to assist with the dislocation of vertebrae (Jaksch, 1981; Martin, 2015a).

Research has previously investigated CD and onset of insensibility and death. One study investigating euthanasia methods for broilers and layers used the loss of nictitating membrane reflex to demonstrate that insensibility occurs in under 3.5s (Martin et al., 2016). This study also found that with CD death occurred within 95s for broilers and 114s for layers, as determined by the end of cloacal movement, and had 100% success rate (Martin et al., 2016). A second study used EEG to establish that insensibility occurred 3.2s after euthanasia attempt and found CD to have a 100% success rate (Martin et al., 2015). Recent research by Jacobs and colleagues comparing CD and KED found it took 8 to 15s for the nictitating membrane reflex to cease, indicating onset of insensibility, and around 90-150s for musculoskeletal movement to cease, indicating onset of death, in broilers euthanized at 36, 42 and 43 days of age (Jacobs et al., 2019). Research investigating multiple methods for the euthanasia of turkeys at 1 and 3 weeks of age, determined via the loss of the nictitating membrane reflex, that insensibility occurred at 43s (Erasmus et al., 2010a) and 55s (Woolcott et al., 2018b) and via the cessation of convulsions that death occurred at 138s for turkeys (Erasmus et al., 2010a). Both studies also found that CD had a 100% success rate with young turkeys (Erasmus et al., 2010a; Woolcott et al., 2018b).

1.4.2.2 Mechanical cervical dislocation

A number of devices are commercially available for mechanical cervical dislocation. Mechanical cervical dislocation devices (MCD) have been designed to result in euthanasia via the same mechanisms as MCD, via a luxation of the spinal column, a severing of the spinal cord and a rupturing of the carotid arteries. The stretch and twist motion that is vital with MCD is often not replicated by mechanical devices, which can lengthen the time to insensibility and death and reduce the likelihood of success. Many of the available devices have been shown utilize force to separate vertebrae by driving a blunt edge between two vertebrae causing a distance between them, rather than the stretch or twisting motion (Gregory and Wotton, 1990; Sparrey et al., 2014; Martin, 2015). Some of the devices available or previously investigated for cervical dislocation in the literature, such as the Burdizzo (Erasmus et al., 2018a) and the Semark pliers or Humane Bird Dispatcher (Gregory and Wotton, 1990; Sparrey et al., 2014), have been shown to use forceful separation of vertebrae rather than a stretch and twist motion. The Humane Bird Dispatcher, previously called the Semark pliers, were studied by Gregory and Wotton (1990). They found that as the device worked via crushing; there was no severing the carotids arteries, that in 21% of attempts the spinal cord was not severed, and that it results in death via asphyxia when used to euthanize broilers. Using EEG and the loss of a visually evoked response, the study determined the time to brain death exceeded three minutes with the Human Bird Dispatchers, and that it was longer than the 1¾ minutes it took for brain death to occur with manual cervical dislocation (assisted by a kill cone) (Gregory and Wotton, 1990). Erasmus and colleagues (2010a), studied the Burdizzo as an MCD tool for turkeys. They found that the birds were sensible for an extended period, with nictitating membrane reflex being present until 106s (just under 2 minutes) after euthanasia attempt. The presence of gasping during this period suggest possible distress resulting from crushing occurring with the Burdizzo (Erasmus et al., 2010a). When compared to CD, the study found a longer duration of eye reflexes and thus a longer time to onset of insensibility with the Burdizzo (Erasmus et al., 2010a). Devices such as these are not appropriate for poultry euthanasia, as instead of causing the desired cerebral ischemia, they often result in asphyxia and are likely to result in neck crushing. When crushing occurs it often means the carotid arteries do not rupture, and the blood supply is not cut off, hindering the possibility of cerebral ischemia occurring and increasing the time to insensibility and death (Gregory and Wotton, 1990; Sparrey et al, 2014; Martin, 2015; Martin et al., 2016).

Crushing can also lead to fractures in the vertebral column. Although it is not known if these specific fractures are painful, other bone fracture types have been established as acutely painful in birds (Nasr et al., 2012). Any dislocation method in which crushing occurs whilst the bird is sensible have been found unacceptable by the AVMA (2013). There is also an increased risk that when the devices are applied incorrectly there is a higher occurrence of crushing injury to the bone and surrounding tissues, as well as less severe damage to the spinal cord and the occurrence of incomplete severance of the spinal cord (Gregory and Wotton, 1990; Martin, 2015).

The Koechner Euthanasia Device, KED, is a newer device available for mechanical cervical dislocation. The device is specifically designed to mirror CD, by using a shearing and twisting motion to luxate the vertebrae and sever the carotid arteries and spinal cord, resulting in insensibility and death from ischemia (Koechner et al., 2012). There are four different models of the KED available for use with multiple poultry species, and each specified for different sizes or weight classes of birds (Koechner et al., 2012; Chicken Farmers of Canada et al., 2015). Recent research by Woolcott and colleagues (2018a), investigated the use of the KED device with turkeys of varying ages. Recent research by Jacobs et al. (2019) showed that for broilers at 36, 42 and 43 days of age, the time to insensibility and death was longer with the KED compared to CD. At present, there is no research available on the use of KED with broilers before 36 days of age.

1.4.3 Gaseous euthanasia

Gaseous euthanasia involves exposing an animal to a high concentration of inhalants that will result in a loss of consciousness and death (Galvin, et al., 2005; Raj et al., 2006; AVMA, 2013). The most common inhalants are argon (Ar), nitrogen (N_2), and carbon dioxide (CO_2) (Galvin et al., 2005; AVMA, 2013), and these can be used either in a pure form or in a mixture (Raj et al., 2006). The inhalation of the inert gases Ar and N_2 will displace available O_2 and thus cause death by hypoxia (AVMA, 2013), while the inhalation of CO_2 induces insensibility and death via both hypoxia and hypercapnia. One of the most widely utilized inhalants is CO_2, due to it having possible anaesthetic effects, as well being inexpensive, easy to obtain, it does not result in a buildup of toxic residues in the animals and is relatively safe for workers (HAS, 2006; AVMA, 2013). With on-farm or hatchery gaseous euthanasia, the exposure to the inhalant during this type of euthanasia can be either by gradually introducing the gas into the euthanasia

chamber, or by pre-filling a chamber with an inhalant and then immersing the animal into the pre-filled chamber (Close et al., 1996; Galvin et al., 2005). With gradual induction, as the concentration of gas increases, the bird will lose consciousness, and eventually death will occur, whilst with immersion concentration, there is a rapid induction of insensibility and death in response to the sudden increase in gaseous inhalant inspired (Hawkins et al., 2006).

1.4.3.1 Gaseous euthanasia with carbon dioxide

With gaseous euthanasia involving CO_2, the increased CO_2 in the environment will mean that inhaled air will contain increased concentrations of CO_2 and decreased concentrations of O_2. The reduced O_2 will cause hypoxia, whilst the increased CO_2 will cause hypercapnia (AVMA, 2013; Terlouw et al., 2016a). Hypercapnia is produced by a rise of CO_2 inhaled and reduced ability to expire CO_2, and will result in increasing concentrations of CO_2 in the bloodstream, leading decrease in blood pH as blood acidifies (Gerritzen, et al., 2013; Cors, et al., 2015; Terlouw et al., 2016a). The acidic nature of the blood causes acidification of the cerebral spinal fluid (CSF). The CSF acidification is detected by chemoreceptors in the medulla, which will increase respiration rate. As the respiration rate increases, the concentration of CO_2 respired continues to increase and further decrease the blood pH, eventually leading to respiratory depression. Acidification of the CSF will also lead to an acidification of the brain cells, resulting in a depression of brain activity and a loss of consciousness (Terlouw et al., 2016a). The reduced activity in the brain and the respiratory system and the resulting hypoxia leads to loss of brain, respiratory and cardiac function, followed by death (Gerritzen et al., 2013; Cors et al., 2015; Terlouw et al., 2016a). Hypoxia, or the lack of O_2 in the bloodstream, will result in death as the reduction in O_2 available in the blood, will mean there is reduced O_2 available for the cells to use for energy metabolism. If there is insufficient O_2 for energy production, the cell will undergo a metabolic crisis resulting in cell death (ESFA, 2004). Global occurrence of extensive cell death will render the vital process non-functional, and result in a loss of respiratory, cardiac and brain function and followed by death (ESFA, 2004).

The inhalation of high concentrations of CO_2, such as with gaseous euthanasia, will result in a loss of consciousness and death. However, there are welfare concerns associated with CO_2 euthanasia due to it having some distressful properties and because the onset of insensibility is not immediate. This means there is a period of time from initial gas exposure prior to

consciousness being lost in which the bird may be conscious to any distress or discomfort associated with CO_2 exposure and euthanasia (Raj et al., 2006; Gerritzen et al., 2013). The respiratory depression during CO_2 euthanasia creates dyspnea. Dyspnea, or breathlessness, is the feeling of straining or having difficulty breathing and breathing discomfort, and is reported as being unpleasant and possibly distressful to poultry (Hawkins et al., 2006; Raj et al., 2006). Furthermore, CO_2 is an acidic gas and when in contact with the mucosa (water) will form carbonic acid, which is believed to cause distress and pain (Lambooij et al., 1999; Hawkins et al., 2006; Turner et al., 2012). Research performed with rodents suggests that with certain induction methods, it may be possible to induce insensibility with CO_2 concentrations below those that have been shown to be aversive (Burkholder et al., 2010; AVMA, 2013). However, there is insufficient evidence whether it is possible to induce insensibility prior to the CO_2 levels causing distress (Webster and Fletcher, 2001; Gerritzen et al., 2004; McKeegan et al., 2006).

1.4.3.2 Neonate euthanasia

Within the commercial poultry industry, there is a need for the euthanasia of day-old chicks and poults directly post-hatch. Ordinarily, euthanasia is performed when the chicks and poults are unviable, malformed or moribund, or because there is currently little requirement for male layer chicks within poultry flocks. Maceration is a common method of euthanasia, that both the Canadian Codes of Practice for Poultry (NFACC, 2016a) and the American Veterinary Medical Association (AVMA, 2013) list as an acceptable-with-condition method for chicks and poults up to 72 hours of age. Despite this fact, the use of maceration is becoming unfavourable for perceived esthetic reasons, and hatcheries are investigating the use of CO_2 gas as an alternative for euthanasia of chicks and poults (Gurung et al., 2018).

There is a lack of scientific research available of the proper methodology for using CO_2 for humane euthanasia of neonate chicks and poults. Neonatal poultry have been shown to be more resistant to hypercapnia and anoxia, thus having a higher tolerance to higher concentrations of CO_2 (Jaksch, 1981). This resistance has been attributed to a compensation mechanism developed by the embryo *in ovo* to deal with the high CO_2 environment (14%) of the egg prior to hatch (compared to normal atmospheric CO_2 of 0.04%) (Jaksch, 1981), and the high arterial pCO_2 prior to hatch, which has been shown to reach 60 mm Hg (increased from 20 mmHg at day 10 of incubation) (Freeman and Vince, 1974). The ability to tolerate high CO_2 concentrations means

newly hatched poultry require higher CO_2 concentrations for euthanasia than older or adult birds (Jaksch, 1981; Gurung et al., 2018). Previous research on gaseous euthanasia with day-old chicks looked at CO_2 euthanasia via immersion into 60% CO_2 in residual air (Raj et al., 1992) and 90% CO_2 in residual air (Raj and Whittington, 1995). The studies found that chicks exposed to 90% CO_2 died within 2 minutes, whilst the majority of chicks exposed to 60% CO_2 were not dead after 5 minutes of exposure (Raj et al., 1992; Raj and Whittington, 1995). When compared to adult hens that died within 2 minutes of exposure to 49% CO_2, it was concluded that neonates required a longer exposure time and higher CO_2 concentrations for successful euthanasia in comparison to adult birds (Raj et al., 1992). A recent investigation comparing alternative euthanasia methods for day-old male layer chicks looked at birds' physiological stress response and time to insensibility and death (Gurung et al., 2018). In the study, gaseous euthanasia with a final concentration of 75% or 90% CO_2 was compared to gaseous euthanasia with 100% N_2 and Low Atmospheric Pressure Stunning. Because no difference was found in the stress response between treatments, and the CO_2 treatments resulted in the shortest time to insensibility and death, gradual introduction of CO_2 to a final concentration of 75% was deemed sufficient for humane euthanasia (Gurung et al., 2018). The study also found that CO_2 concentrations of 25% and 50% were not sufficient to result in death (Gurung et al., 2018). The current hatchery practice for euthanasia of day-old cull chicks with CO_2 is stated as using concentrations of 60-70% and an exposure time of 5 minutes (AVMA, 2002; Hester, 2005). However, there is great variation in the recommendations made in the literature regarding the concentration, exposure time and flow rate. Furthermore, to the best of the author's knowledge, there is no research with neonates on distress associated with CO_2 euthanasia.

1.5 Dehydration

1.5.1 Dehydration and osmoregulation

Osmoregulation or the maintenance of a correct balance of water and electrolytes within the body is a constant and vital process within a healthy bird. Water is being lost at a consistent rate from the body during various metabolic processes, and it needs to be replenished at an equal rate to ensure the body can maintain its physiological functioning (Goldstein and Skadhuage, 1999; Ilheukwumere and Herbert, 2003). When there is insufficient water intake, the volume of extracellular fluid within the bird is reduced, leading to an imbalance of water to electrolytes, and an increase in plasma osmolality, as levels of sodium and other electrolytes increase while

the extracellular fluid volume is reduced (Goldstein and Skadhuage, 1999). The rise in extracellular osmolality will also cause intracellular dehydration. With cellular dehydration, the volume of intracellular fluid is reduced, as the cellular water moves out of the cell into the bloodstream in order to restore the fluid balance (Goldstein and Skadhuage, 1999). Both intra- and extracellular dehydration will stimulate processes to restore the deficit in the osmotic balance (Goldstein and Skadhuage, 1999). The primary restorative mechanism to increase fluid volume is to increase water intake. In birds, the principal method of water intake is drinking, with drinking water providing 70-75% of water intake and birds consuming approximately 5% of their body mass on a daily basis. Other routes of water intake are dietary water, which provides 12-15%, and metabolic water, which provides about 15% of water intake (Goldstein and Skadhuage, 1999; Leeson et al., 2007; van der Klis and de Lange, 2013; Reece, 2015a). In response to an osmotic imbalance, the bird is stimulated to increase water consumption to restore the water deficit. The other restorative mechanism used by birds is water conservation or retention. Water conservation aims to increase fluid volume by reducing the amount of fluid the bird loses. Water loss occurs continuously during normal physiological processes, primarily via excretion or evaporation and evaporative loss from exhaled gases (Goldstein and Skadhuage, 1999; Leeson et al., 2007; van der Klis and de Lange, 2013; Reece, 2015a). With renal excretion, water is lost in the form of urine, as the body eliminates waste, excessive electrolytes, solutes and water. In order to conserve water during excretion, the avian kidney can reduce water loss during excretion by controlling the glomerular filtration rate and tubular reabsorption to maximise water reabsorption (Goldstein and Skadhuage, 1999; McWhorter et al., 2009). Birds have also developed a mechanism that allows for the reabsorption of water and solutes in the hindgut with water and electrolytes being reabsorbed from the urine, in the copradeum, cloaca and caecae (Goldstein and Skadhuage, 1999; McWhorter et al., 2009). Water is also lost via evaporation from the skin and respiratory tract. Respiratory evaporation is a significant component of avian thermoregulation, and as respiration is modulated in order to control body temperature, this will also affect water lost via evaporation (Goldstein and Skadhuage, 1999). Birds have developed mechanisms to counteract osmotic imbalance in order to return to osmotic balance. Dehydration is an osmotic imbalance, classified by a deficit of water in the body, in which the volume of fluid lost exceeds the volume of fluid gained (van der Klis and de Lange, 2013; Reece, 2015a). During dehydration, birds will try to buffer the misbalance of water to

maintain a constant plasma volume, by moving body water between the fluid compartments to minimise the damage of water loss (Leeson et al., 2007; Chikumba et al., 2013; Reece, 2015a). If this is not sufficient to compensate for the water loss, then there will be a shift of intracellular fluid to the plasma (Leeson et al., 2007). A loss of over 10% of total body water has been reported as severe for the majority of animals, including poultry (Reece, 2015a) and can have adverse effects on animal health.

1.5.2 Pathophysiology of avian dehydration

When a bird is dehydrated, the effect of the total body water loss is systemic. As the total fluid volume decreases, the blood volume will also decrease. With this blood volume decrease, the concentrations of electrolytes, solutes and blood components will increase, resulting in an increased blood osmolality as well as increased blood viscosity (Swayne and Radin, 1991). The hypovolemia resulting from dehydration will have further effects throughout the body, such as increasing heart rate, respiration rate and body temperature, as the body aims to return to homeostasis (Joshi and Link, 1971). If water levels are not restored this will lead to health issues such as increased susceptibility to heat stress, metabolic acidosis, arrhythmias such as bradycardia, circulatory failure, damage to the nervous system (Leeson et al., 2007) and toxaemia (Bierer et al., 1965; Leeson et al., 2007). It has also been shown to cause pathologies like nephrosis and visceral gout, proventriculitis (Bierer et al., 1965) and cyanosis of the comb (Bierer et al., 1965; 1966).

On a cellular level, the reduced blood volume and increased blood osmolality will start osmosis and water diffuses from the cell into the blood vessels to restore osmotic balance. The loss of intracellular water will result in cell shrinkage (Graham, 2016). If dehydration is severe, this cell shrinkage can lead to organ damage and eventually organ death. Weight loss will also occur as the body dehydrates, with approximately 5% of body weight being lost daily (Arad, 1983). This weight loss can be attributed to a reduction of feed intake. Birds reduce the intake of food in an attempt to reduce additional intake of sodium and other electrolytes, to minimise the already higher proportion of solutes and electrolytes within the body system (Arad, 1983; Ilheukwumere and Herbert, 2003). Reduced feed intake and weight loss will result in reduced productivity, by stunting growth and negatively impacting feed conversion ratio as the birds reduce metabolic efficiency and feed intake (Castro et al., 2009; Viola et al., 2009; Vanderhasselt

et al., 2013; Iheukwumere & Herbert). Death is likely to occur if over 45% of body weight is lost (Leeson et al., 2007).

1.5.3 Dehydration in a poultry flock

The monitoring of the water intake of a poultry flock is a tool utilized on-farm to judge the health and productivity of a flock. This is done as changes in water consumption can be an early indicator of issues with management, water quality or health status of the flock (Manning et al., 2007). With water intake being monitored on a regular basis, flock-wide dehydration is an uncommon occurrence unless resulting from incidents like equipment malfunction or inappropriate management causing flock-wide water deprivation (Bierer et al., 1964; Manning et al., 2007). Dehydration occurs with a higher frequency on an individual bird basis within a poultry flock, with potentially a high proportion of daily mortality being attributed to dehydration (Butterworth et al., 2002). With individual birds suffering from dehydration, water deprivation is attributed to an inability to reach or operate the drinker or water source, and consequently consume the required water amount (Butterworth et al., 2002; Manning et al., 2007; Sprenger et al., 2009; Rault et al., 2016). An inability to access water sources can be the result of a myriad of issues, and it is often associated with bird size, lameness and disease (Butterworth et al., 2002; Sprenger et al., 2009; Rault et al., 2016). It is common practice within a poultry flock for the drinker lines to be raised as the birds grow. The raising of the drinkers can mean they can be raised to a height which smaller birds or runts are unable to reach, thus hindering their access the water source (Butterworth et al., 2002; Gregory, 2004; Savory, 2010). Lameness or locomotion problems or injuries that inhibit movement hinder a bird's ability to walk to and operate drinkers, thus resulting in dehydration (Butterworth et al., 2002; Sprenger et al., 2009; Rault et al., 2016). Lethargy and other negative symptoms of disease can also create difficulties for the birds to access the water source and cause water deprivation and dehydration (Gregory, 2004; Butterworth and Weeks, 2010; Savory, 2010). If birds are unable to access the water source for an extended period of time, this will lead to chronic dehydration and eventual mortality. Dehydration has a significant negative impact on bird welfare, as it causes thirst and discomfort, and when dehydration is severe, also causes ill health and distress (Sprenger, 2009, Vanderhasselt, 2014 & 2013, Rault, 2016). Furthermore, dehydration is often found as a cause for euthanasia or as a comorbidity to the reason for culling, thus it vital for bird welfare to minimise dehydration and ensure timely culling.

1.6 Conclusion

Euthanasia is often required on-farm or in the hatchery to end suffering or potential suffering of birds that are ill, moribund or unviable. Physical methods of euthanasia, such as the NPCD and MCD, are available options for on-farm euthanasia. However, to determine their appropriateness for the use with broilers, more knowledge is needed regarding their ability to rapidly disrupt brain function, and to reliably result in irreversible insensibility and death without causing additional suffering, pain or distress. Furthermore, knowledge on the traumatic injury and physical damage produced by the methods and the relationship of this damage to the efficacy or appropriateness of the methods is also necessary. Research has shown that NPCD, and the Zephyr specifically, are appropriate for the on-farm euthanasia of turkeys. However, research is needed to understand whether the Zephyr is appropriate for use with broilers at different ages. Previous studies investigating MCD, have shown that devices that result in crushing are inappropriate for use. Information on whether KED results in crushing with broilers is unavailable; thus, research is needed to understand mechanisms underlying euthanasia when using the KED, as well as the latency to insensibility and death, to establish whether it is appropriate for broilers. The data from this research will increase scientific understanding of on-farm euthanasia methods for broilers and provide information on their efficacy and humaneness to aid in the euthanasia decision-making process.

Gaseous euthanasia, with CO_2 as an inhalant, is one method of euthanasia used in hatcheries for the disposal of cull chicks on day of hatch. At present, there is very little research available on the best methodology for using CO_2 to euthanize neonates. In order to determine the best method of using CO_2, information is needed on the induction method, exposure duration and minimum CO_2 concentrations, and how these affect the latency to distress, insensibility and death. The data from this research will provide information to establish the most humane and efficacious manner to use CO_2 for the euthanasia of neonate cull chicks. In addition, this research aims to understand the influence of dehydration on the efficacy of euthanasia methods. Within a poultry flock, birds that require euthanasia are often also found to be suffering from dehydration. Often as a direct result or comorbidity to the main reason for culling, such as an injury, lameness, or lethargy, which hinders the bird's ability to reach drinkers resulting in dehydration. Little is known on the effect of dehydration on the efficacy of euthanasia, and research is needed to address this issue.

1.7 Objectives

The primary objective of this thesis was to assess the efficacy and welfare impact of euthanasia methods for broiler chickens and evaluate their ability to induce instantaneous insensibility and consistently result in death with minimal pain and distress.

To accomplish this, specific objectives included:

- To determine whether the traumatic injury and damage produced by three on-farm euthanasia methods is sufficient to result in insensibility and death and whether the methods cause insensibility and death via the intended mechanism.
- To compare the ability of three commercially available on-farm euthanasia methods at reliably inducing insensibility and death without additional suffering.
- To determine the impact of water deprivation on the euthanasia process and the latency to insensibility and death.
- To determine the impact of water deprivation on the efficacy of three commercially available on-farm euthanasia methods.
- To examine the efficacy of different gas induction techniques for CO_2 euthanasia of neonate broilers at inducing insensibility and death.
- To evaluate distress associated with different gas induction techniques for CO_2 euthanasia of neonate broilers.
- To evaluate the effect of CO_2 immersion concentration on the efficacy of gaseous euthanasia for neonate broilers.
- To determine how the age of birds affects the efficacy of gaseous euthanasia.

1.8 Hypotheses

The hypotheses of this study include:

Hypotheses regarding on-farm euthanasia:

1. Not all the on-farm euthanasia methods will be appropriate for use at all ages. Depending on the age of the birds, the euthanasia methods may not result in sufficient physical damage needed for successful euthanasia, or may result in excessive physical damage.
    - The size of the teeth of the Koechner Euthanasia Device may be too large to fit between vertebrae of the youngest and smallest birds, inhibiting it from severing the spinal cord and causing additional damage to the spinal vertebrae.
    - The pressure and velocity of the Zephyr may be too high for the less ossified skulls of the smallest and youngest birds, resulting in excessive damage, which may be aesthetically unpleasant for the operator.
2. The non-penetrative captive bolt (Zephyr) will result in a shorter latency to onset of insensibility and death compared to the two cervical dislocation methods.
    - The Zephyr utilizes concussive force to disrupt brain function leading to an instantaneous unconsciousness and rapid death.
    - Cervical dislocation methods result in insensibility and death via cerebral ischemia, as the development of cerebral ischemia is not instantaneous; the time to insensibility and death will be lengthened.
3. Water deprivation will increase the time to onset of insensibility and death with euthanasia.
    - Water deprivation will result in physiological changes throughout the body that will affect the process via which consciousness is lost and death occurs with on-farm euthanasia methods.

Hypotheses regarding hatchery euthanasia:

1. A fast gradual CO_2 flowrate will have the least negative welfare impact on cull broiler chicks.
    - The rapid increase in CO_2 with a fast flow rate will result in rapid insensibility and death.
    - It will allow for consciousness to be lost prior to the CO_2 concentration causes distress.
2. Gaseous euthanasia via immersion into 100% CO_2 will result in the shortest latency to insensibility and death.
3. Immersion induction with a CO_2 concentration of 80% will be sufficient to induce rapid insensibility and death with a short exposure to distressful CO_2 concentrations.
    - Death occurs around 75% CO_2 when neonates are euthanized via gradual induction.
4. Latency to the onset of insensibility and death will decrease as birds age.
    - Neonates have a higher tolerance to CO_2, and as broilers age this tolerance will decline and the time taken for CO_2 to result in insensibility and death will be reduced.

2.0 Chapter Two - Evaluating the efficacy of three on-farm euthanasia methods for broilers throughout the production cycle on behavioural reflex responses for insensibility and death, anatomical pathology, radiography and histology.

The objective of this work was to examine different euthanasia methods for broilers by comparing their efficacy and welfare impact. Chapter 2 aims to evaluate commercially available euthanasia methods for use on-farm. To understand the efficacy and welfare impact of the euthanasia methods, this chapter used radiography, gross pathology and histology to evaluate the traumatic injury and damage produced, as well as studying behavioural indicators to assess the methods ability to induce a rapid insensibility and death.

2.1 Abstract

Euthanasia is common to end the suffering of disease or moribund broilers with diminutive chances of recovery. This study aims to evaluate the efficacy of commercially available on-farm euthanasia methods at inducing insensibility and death. The first phase assessed the damage and trauma produced by each of three methods to induce euthanasia on the cadavers of cull birds (n=180); the second phase evaluated behavioural reflexive indicators to measure latency to insensibility and death of cull broilers (n=240). Manual cervical dislocation (CD), mechanical cervical dislocation with a Koechner Euthanasia Device (KED), and a non-penetrative captive bolt (Zephyr), were evaluated for use on broilers at 7, 21 and 35 days of age. Radiograph, gross pathology and histology were used to score the trauma to the cranial – cervical region resulting from the euthanasia methods. Latency to insensibility was measured by time to absence of pupillary light, palpebral blink, and nictitating membrane reflexes. Time to death was measured by latency to occurrence of feather erection and cessation of rhythmic breathing, cloacal winking and convulsions. Behavioural data were analysed for the main effect of euthanasia method as an RCBD (block=farm; bird as experimental unit) with PROC MIXED (SAS 9.4). Score data were Friedman rank transformed (PROC RANK), then tested with an analyses of variance (PROC MIXED) for the main effect of euthanasia method (farm as block). Differences were considered significant when $P \leq 0.05$. Scores for subdural hemorrhaging, subcutaneous hemorrhage on the head, and skull fracture were higher for birds euthanized by Zephyr than birds euthanized by CD or KED. Both CD and KED resulted in high subcutaneous hemorrhage on the neck scores at 21 and 35 days, indicating severing of the carotid arteries. Birds euthanized by CD had 98% of spinal cords completely transected and those euthanized by KED had 81% of spinal cords transected. The number of fractures in the vertebral column was higher with the KED than CD at 21 and 35 days of age. Indicators of insensibility were lost earliest with the Zephyr, than with CD and took longest to be lost with KED, at 21 and 35 days. Cessation of cloacal winking and convulsions occurred earlier with birds euthanized by CD, compared to birds euthanized by either the KED or the Zephyr. The post-mortem data indicate that the Zephyr caused sufficient concussive damage to the brain to result in rapid insensibility, and that CD consistently resulted in a severing of the spinal cord and the carotid arteries, whilst the KED did not always result in a complete severing of the spinal column and had more fractures present. The ante-mortem data indicates that the Zephyr resulted in the shortest latency to insensibility whilst CD resulted in the

shortest latency to death for birds of 21 and 35 days of age. At seven days of age, CD also resulted in the shortest time to death. Overall, all of the euthanasia methods studied where efficacious at inducing insensibility and death, but the shortest time to insensibility occurred with the Zephyr and the shortest time to death occurred with CD.

2.2 Introduction

An important component of good broiler management is humane and timely euthanasia. Throughout all the stages of the production cycle, euthanasia of broilers is often necessary due to illness, injury or when birds are otherwise unviable. Canadian animal care guidelines for poultry require that producers take appropriate action to end the life and suffering of an animal if they are unfit, injured, sick or moribund and have no prospect of recovery (NFACC, 2016a). Euthanasia benefits the individual animal by ending pain and suffering, but also improves the group welfare and productivity, as well as providing economic benefit to the producer (Turner and Doonan, 2010). The act of euthanasia or humane killing allows the ending of an animal's life in a manner that minimizes or eliminates pain or distress (Erasmus et al., 2010a,b,c; AVMA, 2013; Thornber et al., 2014; Cors et al., 2015; NFACC, 2016a), and the efficacy or humaneness of a euthanasia method is often based on its ability to result in a rapid, irreversible loss of consciousness (insensibility) and death (Erasmus et al., 2010a,c; Thornber et al., 2014; Cors et al., 2015; NFACC, 2016a).

Consciousness allows the birds to have an awareness of the environment including internal and external stimuli (Erasmus et al., 2010b; Terlouw et al., 2016a). This is important in euthanasia, as a state of consciousness allows for the perception, interpretation, integration and response to sensory information that the bird may experience as painful or distressful. To minimise the bird's ability to experience negative welfare during euthanasia, it is vital that there is a rapid loss of consciousness or onset of insensibility with euthanasia and stunning (Benson et al., 2012 a,b).

Insensibility, or the state when consciousness has been lost, is an impairment to brain function resulting in a state of unawareness, in which there is an inability to respond to normal stimuli and a disruption to the integration of sensory information (Erasmus et al., 2010b). The induction of insensibility means the animal is insensitive to normal stimuli and no longer able to process sensory information (Terlouw et al., 2016a), and thus it cannot be aware of its surroundings or experience pain, distress or other negative affective states (Erasmus et al., 2010b; Benson et al., 2012 a,b; Terlouw et al., 2016a). Insensibility occurs when there is a reversible or irreversible dysfunction of the brain structures that are essential to consciousness (Terlouw et al., 2016a). Within the avian brain, the key structures for consciousness are the

brainstem, thalamus and pallium (Renier et al., 2005; Butler and Cotterill, 2006; Erasmus et al., 2010b; Martin, 2015; Terlouw et al., 2016a). The brainstem mediates consciousness and maintains the level of arousal (Erasmus et al., 2010b; Terlouw et al., 2016a). The thalamus and pallium allow for sensory information to be perceived and integrated, and this information is translated into conscious sensory experience (Butler and Cotterill, 2006; Adams and Sheridan, 2008; Terlouw et al., 2016a). A functional and active consciousness requires the pallium and thalamus for conscious perception and subjective experience, and the brainstem and reticular formation for the regulation of sensory information transfer and maintaining awakeness (Renier et al., 2005; Verhoeven et al., 2015). Disruption to the normal functioning of these structures leads to insensibility (Erasmus et al., 2010b; Terlouw et al., 2016a). This dysfunction can be due to direct insult to these regions, hypoxia or physical disruption by neuron depolarisation or hyperpolarisation, and concussion (Erasmus et al., 2010b).

For humane euthanasia, insensibility should be rapidly or immediately followed by death (Thornber et al., 2014; Cors et al., 2015). Death is a process in which an animal transitions from being alive to a final state of dead, when all the parts of the animal and the animal as a whole have ceased to function (Adams and Sheridan, 2008; Martin, 2015). In reference to euthanasia, death is often described as involving three components: a cessation of brain function, a cessation of respiratory and circulatory function and a cessation of cardiac function (Thornber et al., 2014; Cors et al., 2015; Martin, 2015). The brain is fundamental to living and the destruction or rendering non-functional of the brain results in brain death. During brain death, cerebral activity is abolished, and the respiratory and cardiac activity control centres fail. There is also an inability to induce spontaneous or reactive behaviours and an absence of brainstem and deep tendon reflexes (Sandercock et al., 2014; Martin, 2015; Terlouw et al., 2016a). The brainstem is key to maintaining life and its disability results in brain death. Vital life-sustaining functions throughout the body are controlled by the brainstem, and include functions such as the conductance of motor and sensory information between the higher brain centres and body, the regulation of respiratory and cardiovascular function, central nervous control and maintaining consciousness (Martin, 2015; Terlouw et al., 2016a; Woolcott, 2017). When the brainstem is rendered non-functional, the respiratory and circulation systems will cease, the rest of the processes and organs within the animal will soon follow and life will end. This importance of the

brainstem in sustaining life is the reason why it is often the main target for destruction in many euthanasia methods.

Euthanasia methods are often evaluated on their efficacy or humaneness in terms of their ability to induce a rapid loss of consciousness and death. One method utilized to measure insensibility and death, and which can be performed in a whole range of situations (on-farm, in a laboratory setting or at a slaughterhouse) that has been validated and widely used in various research studies is measurement of involuntary behaviours/behavioural or reflexive indicators (Gerritzen et al., 2004; Erasmus et al., 2010a,b,c; Sandercock et al., 2014; Martin, 2015; Woolcott, 2017; Woolcott et al., 2018a). Cranial reflexes or brainstem reflexes are indicators of insensibility and/or brain death, as they give insight into the functioning of the brainstem by evaluating the functional link between the brainstem and cranial nerves. These nerves originate from the brainstem and they relay information to the body, particularly the cranial-cervical region, without control or involvement from the cortex and spinal cord (Erasmus et al., 2010b; Verhoeven et al., 2015; Terlouw et al., 2016b). Disruption to the brainstem will result in impairment of cranial nerve functioning and an absence of brainstem reflexes. One of the brainstem reflexes is the corneal or palpebral blink reflex. The sensory information about the approach/touching of the cornea or eyelid is transmitted via the trigeminal nerve (cranial nerve V) to the brainstem and stimulates a motor response by facial nerves (cranial nerve VII), which in turn shuts the eyelid and results in a blink (Erasmus et al., 2010b; Martin et al., 2016; Terlouw et al., 2016b). The nictitating membrane, or the bird's third eyelid, can also be used as a brainstem reflex indicator. The sensory information from the touch or approach of the cornea is carried by the trigeminal nerve, which projects into the brainstem, then inputs to the sixth cranial nerve, or abducent nerve, which elicits the movement of the nictitating membrane horizontally across the eye below the outer lids (Stibbe, 1928; Martin et al., 2016; Terlouw et al., 2016b). Another brainstem reflex is the pupillary light reflex, the contraction of the pupil in response to light exposure, with an absence of response or mydriasis (dilated pupils) indicating insensibility or brain death. The optic nerve (cranial nerve II) controls sensory input, and relays information to the midbrain, stimulating a response of the pupil via the oculomotor nerve (cranial nerve III) (Erasmus et al., 2010b; Martin et al., 2016; Terlouw et al., 2016b). Impairment to the underlying neural circuit will result in a loss of these reflexes, and as the neural circuits responsible for these

reflexes are also responsible for consciousness, the absence of these reflexes indicates brainstem dysfunction and that the animal is unconscious or braindead.

Rhythmic breathing is another involuntary behaviour whose absence suggests that the animal is insensible or dead. The medulla oblongata, a component of the brainstem, houses the respiratory control centre whose nerves project onto the respiratory muscles and are responsible for the autonomic regulation of both inspiration and expiration (Martin et al., 2016; Terlouw et al., 2016b). The alternate activation of muscles responsible for inspiration and expiration results in rhythmic breathing. A cessation or absence of rhythmic breathing shows that the respiratory centre no longer has neurological control over respiration, which can occur with a disruption or rendering non-functional of the reticular formation or the medulla, both key players in consciousness (Martin et al., 2016; Terlouw et al., 2016b).

Convulsions and the cessation of movement have been used in several studies on euthanasia as indicators of death. Convulsions are bi-phasic involuntary, uncontrolled neuromuscular spasms. The first of phase is clonic convulsion, or the spastic movement of the wings and legs, often referred to as wing-flapping and leg paddling. The second phase is tonic convulsions, which involves momentary stillness with muscle rigidity of outstretched wings and legs and final gentle paddling motions (Dawson et al., 2007; Dawson et al., 2009; Erasmus et al., 2010a,b; Woolcott, 2017). Several studies have shown that convulsions are incompatible with consciousness, and the occurrence of convulsions indicates insensibility (Dawson et al., 2007; Dawson et al., 2009; Verhoeven et al., 2015; Verhoeven et al., 2016). The end of the tonic phases has been shown to be an indicator of brain death, or irreversible brain failure (Dawson et al., 2007; Dawson et al., 2009; Erasmus et al., 2010a,b; Martin, 2015). Cloacal movement or winking is the opening and closing of the cloacal sphincter or vent, and an indicator of brain death (Erasmus et al., 2010b, Martin et al., 2016). This indicator is often one of the last reflexes or involuntary behaviours to cease, and therefore may be a conservative indicator of death (Martin et al., 2016). Piloerection, or a sudden global erection of feathers in an insensible bird is another indicator used to evaluate time of death. Although not widely used, other studies have noted the occurrence of feather erection during tonic convulsion and it has been suggested to be a sign of cardiac arrest or hypoperfusion (Heard, 2000; Erasmus et al., 2010b). Thus the occurrence of feather erection suggests a cessation in cardiovascular activity, which could

indicate a cessation of the cardiovascular regulatory centres in the brainstem as a result of brain death, as well as an indicator of absolute death (Casey-Trott et al., 2013,2014).

Percussive non-penetrative captive bolt devices (NPCD) are one common on-farm euthanasia method (Galvin et al., 2005; AVMA, 2013; Sparrey et al., 2014). The mode of action of NPCD is similar to blunt force trauma and aims to result in insensibility and death by physical disruption of the neurons. Both NPCD and blunt force trauma utilize concussive force to interrupt the correct functioning of neurons throughout the brain to disrupt sensory processing and induce insensibility and death (Galvin et al., 2005; Erasmus et al., 2010c; Casey-Trott et al., 2014; Sparrey et al, 2014; Cors et al., 2015; Martin et al., 2016; Terlouw et al., 2016a). NPCD uses non-penetrative bolts that rely on the kinetic energy displayed by the impact of the strike on the cranium to produce concussive effects throughout the brain (Grist et al., 2017), without penetrating the brain (Woolcott et al., 2018a). The impact of the bolt will result in traumatic brain injury as it causes disruption to both the brain structure and function, and both via the initial damage and a cascade of secondary injuries (Gaetz, 2004; Andriessen et al., 2010; Tseng et al., 2011; Xiong et al., 2013; Martin, 2015; Woolcott, 2017). The initial damage occurs from the mechanical disruption of the brain as a direct consequence of the bolt impact, and often involves damage or rupturing of blood vessel (hemorrhage), contusions, axonal shearing and skull fracture (Xiong et al., 2013; Martin, 2015; Woolcott et al., 2018a). The force of the impact on the cranium causes a compression of the tissue localised directly beneath the site of impact resulting in these injuries. This damage at impact site is often described as coup (Martin, 2015; Woolcott, 2017). Contre-coup damage also occurs, with tissue damage opposite the site of impact occurring as the acceleration/deceleration forces produced by the bolt impact causes the brain to move within the cranial vault and collide with the side of the cranium. This collision causes stretching, tearing, compression and deformation of neurons and blood vessels, resulting in contusion, hemorrhage and axonal shearing damage (Shaw, 2002; Gaetz, 2004; Andriessen et al., 2010; Martin, 2015). Further secondary damage occurs because of metabolic, cellular and molecular events initiated by the initial damage. Events such as cerebral oedema, hypoxia or ischemia, increased intracranial pressure, reduced blood flow and glutamate neurotoxicity will lead to further neuron death, tissue damage and atrophy (Gaetz, 2004; Pearce, 2008; Andriessen et al., 2010; Xiong et al., 2013). Contusions have been reported to initiate localised ischemia and oedema which will increase intracranial pressure, whilst hemorrhaging will disrupt blood flow to

the brain causing both cerebral ischemia and altering intracranial pressure (Martin, 2015; Woolcott, 2017). The loss of consciousness with concussion occurs from the interruption of electrophysiological and functional activities of neurons within the brainstem and pallium (Pearce, 2008). With NCPD, the impact of the bolt induces traumatic injury via contusion, hemorrhage, and axonal damage, to the brain areas essential in consciousness thus disrupting their function, and resulting in secondary injury that leads to brain death.

On-farm euthanasia can also be performed by cervical dislocation. Cervical dislocation can be performed either manually or with the assistance of a tool. The goal of cervical dislocation is to cause a separation of the cranium from the spinal column, resulting in a severing or transection of the spinal cord and a rupturing of the carotid artery (AVMA, 2013; Sparrey et al, 2014; Martin et al., 2016). The spinal column is the main neural pathway linking the brain and the rest of the body, by severing the spinal cord cervical dislocation disrupts this transport of information (Gregory and Wotton, 1990; AVMA, 2013; Bader et al., 2014; Sparrey et al, 2014; Martin, 2015; Martin et al., 2016). The cutting off supply of oxygenated blood to the brain, by the rupturing of the carotid arteries, will result in cerebral ischemia that will induce insensibility and death (Gregory and Wotton, 1990; Bader et al., 2014; Sparrey et al., 2014; Martin, 2015). This lack of blood and oxygen in the brain results in a loss of function and cell death, while the low blood pressure in the brain from the reduced blood supply causes a functional impairment of the brain and initiates neurogenic shock (Martin et al., 2016). Research by Martin and colleagues (2016) suggests that even when cervical dislocation does not totally sever the carotid arteries, the narrowing or occlusion of the arteries should reduce blood supply sufficiently enough that it cannot keep up with the metabolic demand for oxygen and still result in cerebral ischemia. The initial mechanical trauma caused by the cervical dislocation method will result in direct trauma or injury to the spinal cord, such as vertebral fractures entering the spinal cord or contusions of the spinal tissue. This trauma then initiates secondary damage via neurogenic shock, hemorrhaging and apoptosis, which impair spinal cord function (Martin, 2015). Insensibility occurs as a result of the loss of blood circulation to the brain and a temporary functional impairment of the spinal cord and brainstem due to massive depolarisations of neurons within these organs (Gregory and Wotton, 1990; Martin, 2015). However, cervical dislocation may not result in an instantaneous loss of consciousness (Gregory and Wotton, 1990; Sparrey et al., 2014).

Manual cervical dislocation (CD) is the dislocation of the neck by hand. This is performed by the stretching and twisting of the neck. The stretching action aims to tear the neck muscles, ligaments, connective tissues and blood vessels, (Martin, 2015), whilst the twisting action by the tipping of head will cause the dislocation of the vertebrae and the transection of the carotid arteries (Sparrey et al., 2014; Martin, 2015). This stretch and twist action results in a stretching of the neural tissue within the brainstem and spinal cord, causing trauma to these regions. It has also been suggested to have a concussive effect of the brain, which causes excessive damage to the brainstem, leading to immediate loss of consciousness (Gregory and Wotton, 1990; Sparrey et al., 2014; Martin et al., 2016). The damage to neural tissue from stretching and twisting causes a sudden increase in ascending electrical neural input which overwhelms the brain, rendering it insensible (Sparrey et al., 2014), as well as initiating biochemical and physical changes which impair the efficacy and functions of the neurons (Martin, 2015). With CD, the aim is a separation high up on the spinal cord, ideally between the skull (C0) and the first vertebrae, altas (C1), or between the atlas (C1) and the second vertebrae, axis (C2) (Sparrey et al, 2014) increasing the likelihood of concussions, which will impair brainstem function and induce biochemical changes within the axons of the local neurons (Martin et al., 2016). Dislocations that occur further down the vertebral column may not result in concussion and an instantaneous loss of consciousness (Gregory and Wotton, 1990; Sparrey et al., 2014). For young birds, CD is sometimes performed using the assistance of a sharp edge, in which the operator uses their thumb to push the chick's neck onto a sharp edge in order to dislocate the neck (Jaksch, 1981; Martin, 2015). With this technique, there is a concern that although the vertebrae are dislocated, the twist and stretch action is not sufficient to sever the carotid arteries and it may result in crushing injuries to the neck and asphyxia (Martin, 2015).

Various mechanical devices have been designed to aid in euthanasia and are designed to work similarly to cervical dislocation by severing the spinal cord and the carotid arteries. A number of these devices have been previously investigated for poultry euthanasia, such as the Burdizzo (Erasmus et al., 2018a), and the Semark pliers or Humane Bird Dispatcher (Gregory and Wotton, 1990; Sparrey et al., 2014). These devices have been found to not result in a rapid insensibility and death, and function by crushing or forceful separation of vertebrae rather than the mechanisms of twisting and stretching seen with CD, which has been found inappropriate by American Veterinary Medical Association (2013) and National Farm Animal Care Council

(2016a). Cervical crushing is an issue, as the carotid arteries do not rupture, thus crushing results in asphyxiation rather than cerebral ischemia, increasing the latency to insensibility (Gregory and Wotton, 1990; Sparrey et al., 2014; Martin, 2015; Martin et al., 2016). Furthermore both crushing or the forceful separation of vertebrae may result in crushing injury to bone and reduce the likelihood of spinal cord damage, and increase the likelihood of fractures (Gregory and Wotton, 1990; Martin et al., 2016; Woolcott, 2017). The Koechner Euthanasia Device (KED) is a mechanical cervical dislocation device that was designed to cause death via shearing and twisting, and not via crushing (Koechner et al., 2012). The device has been designed for use with various species and sizes of poultry, and there are four models available for different sizes of birds (Chicken Farmers of Canada et al., 2015). There has been no research investigating the mode of action and the efficacy of the KED for use with broilers.

The objective of this research was to investigate the efficacy of three on-farm euthanasia methods that are commercially available, including the Zephyr, KED and CD. This was conducted with investigation of each method's ability to induce insensibility and death, evaluating the traumatic injury and damage produced by the methods and whether the techniques are sufficient to result in insensibility and death in a humane way, at three stages of production: starting, growing and finishing. It was hypothesized that at specific ages not all the euthanasia methods would result in the physical damage needed for successful euthanasia or may result in excessive damage. This is because the teeth of the KED devices maybe too large to fit between vertebrae of the youngest and smallest birds, thus not causing a dislocation and severance of the spinal cord, and the pressure and velocity of Zephyr may be too high for the less ossified skulls of the smallest and youngest birds and cause excessive damage. It was further hypothesized that the non-penetrative captive bolt would result in the most rapid induction of insensibility and death, as the device utilizes concussive force to cause brain dysfunction that leads to an instantaneous unconsciousness and rapid death. While with the cervical dislocation methods, insensibility and death occur via cerebral ischemia; as the occurrence of cerebral ischemia is not instantaneous which will lengthen the time to insensibility and death.

2.3 Materials and methods

2.3.1 Ethical Note

The research was conducted with the approval of the University of Saskatchewan Animal Research Ethics Board and all birds were cared for as specified in the Guide to the Care and Use of Experimental Animals Canadian Council of Animal Care (1993, 2009).

2.3.2 Animals

The birds used throughout the research were cull birds from volunteer farms, located within an hour travel time to of the University of Saskatchewan. Farms provided birds from three time periods, once in each production stage, and the birds were obtained from the same flock at 7, 21 and 35 days of age. The birds scheduled for culling were removed from the flock and brought to a separate room on farm for the experimental procedures and data collection. Birds were assigned to euthanasia method treatments at random. The Chicken Farmers of Canada Biosecurity regulations (2014) were met prior to accessing all the farms.

2.3.3 Experimental design and procedure

The efficacy and welfare impacts of the on-farm euthanasia methods at different ages within the production cycle were evaluated in two experiments. The initial experiment evaluated each euthanasia method on cadavers to characterise the extent of physical injury and tissue damage caused by the methods, and to elucidate if this damage was sufficient to cause dysfunction to the structures that control consciousness and vital life function. The methods were also assessed on whether the damage produced would result in insensibility and death without excessive damage and thus suitable for further investigation with live birds. The methods found suitable in experiment one were used in the second experiment and were assessed for latency to insensibility and death, and the extent of physical damage and tissue injury was characterised. Both experiments investigated the efficacy of three euthanasia methods, manual cervical dislocation (CD), mechanical cervical dislocation (KED) and a non-penetrative captive bolt (Zephyr), at three ages of broilers; 7, 21 and 35 days of age.

2.3.3.1 Euthanasia methods

The non-penetrative captive bolt device used was the Zephyr-EXL (Bock Industries, Inc. (BI), Philipsburg, PA, USA). The Zephyr-EXL (Zephyr) is a pneumatic driven captive bolt device, with a nylon head (diameter: 25mm) attached to a cylindrical metal bolt (diameter: 9.5mm). The bolt travels past the barrel of the device by 27.2 mm but does not penetrate the brain of the bird (Casey-Trott et al., 2013; Woolcott et al., 2018a). The Zephyr-EXL was driven by a CO_2 power system (Bock Industries, Inc. (BI), Philipsburg, PA, USA). The system (Figure 2.1) consists of an 800 psi CO_2 bottle, a 120 psi pressure regulator and a short hose that attaches to the Zephyr EXL. The pneumatic pressure of 120 psi results in the bolt moving at 27 m/s or 26 joules. The Zephyr-EXL is applied to the top of the bird's head, just behind the comb. Correct placement is midway between the eyes and ears, just above the cerebrum

Mechanical cervical dislocation was performed using the Koechner Euthanasia Device (Koechner Mfg. Co., INC. Tipton, MO, USA) [Patent number: US 8,152,605]. The KED-S was used for birds at 7 and 21 days of age, whilst the KED-B was used for birds at 35 days of age, as per the manufacturer's specifications (Figure 2.1). The KED is placed around the neck of the bird, the top of the device is directly posterior to the base of the skull and perpendicular to the head and the bottom is just under the cheek of the bird. The device is then closed, dislocating the vertebrae of the spinal cord.

The CD was conducted by a trained adult stock person for days 21 and 35. Birds were held with one hand on the legs and the other hand placed on the neck up against the base of the skull. The head and neck were then dislocated via a stretch and twist motion by applying a downwards pull movement followed by tipping the head up and out (Figure 2.1). For 7 days of the age, the CD was conducted by placing the bird's chin on a sharp edge and pressing firmly on the back of the neck at the base of the skull, thereby dislocating the head and neck. For this a pail, with a 3.5 mm edge, was used.

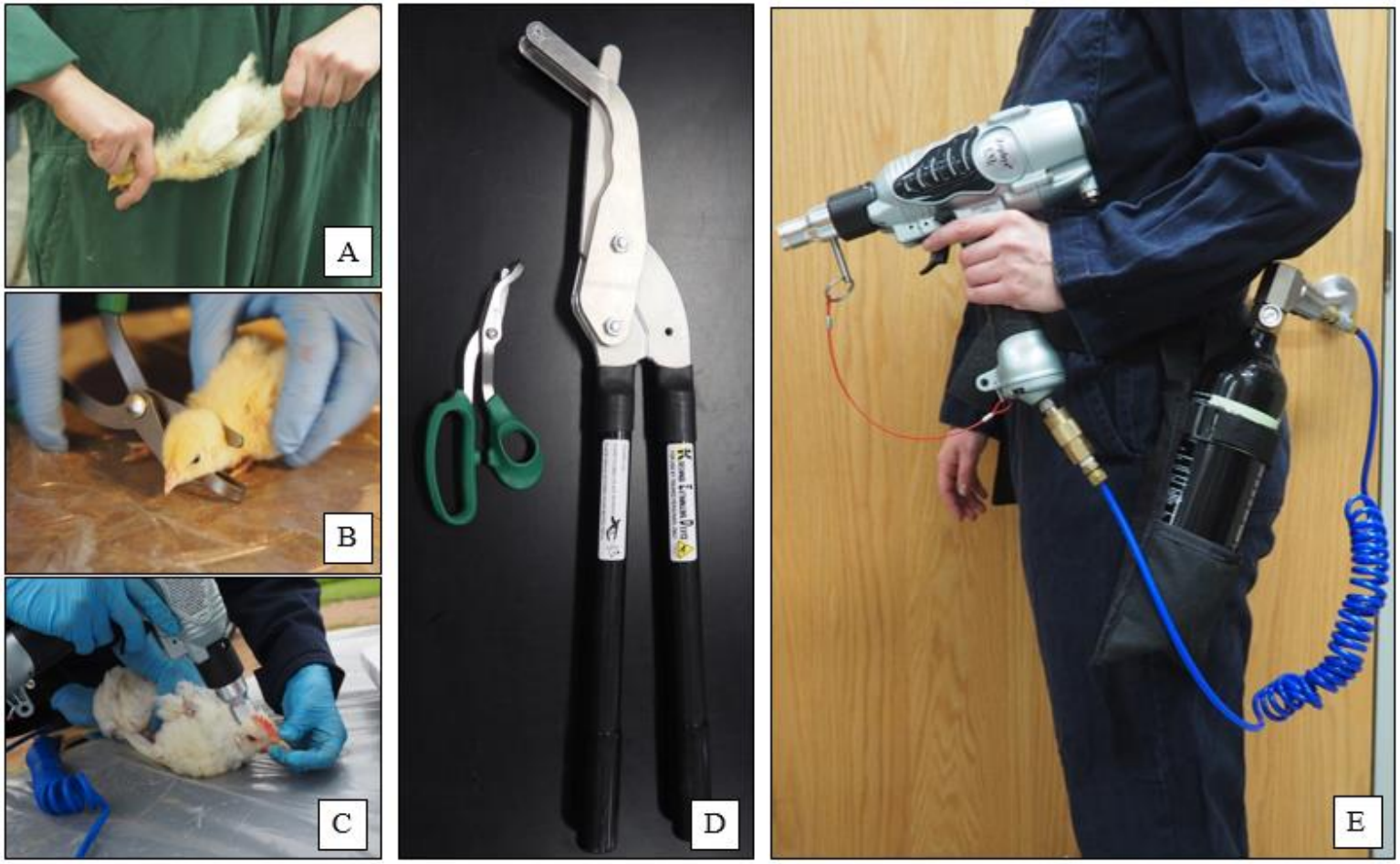

Figure 2. 1. The three on-farm euthanasia methods assessed for use with broiler chickens. Examples of the correct placement and usage during broiler euthanasia for: manual cervical dislocation (A), mechanical cervical dislocation with the Koechner Euthanasia Device (B), and a non-penetrative captive bolt device, the Zephyr (C). (D) The two models of Koechner Euthanasia Device used in this study, the KED-S and the KED-B. (E) The Zephyr, a non-penetrative captive bolt device, combined with the associated CO_2 power system as used in the study.

2.3.3.2 Cadaver experiment

A total of 180 culls birds were used, with 20 birds per age (7, 21 and 35 days) from each of three volunteer farms. For each experimental run, the three euthanasia methods, CD, KED, and Zephyr, were assigned to individual birds. Birds were individually weighed (Starfrit, Atlantic Promotions Inc., Longueuil, QC, CA) and then euthanized using T-61™ (Embutramide, Mebezonium iodide, Tetracaine hydrochloride injectable solution, MERCK, Kirkland, QC, CA) solution. The T-61™ was injected at a dosage of 0.4cc/kg via the jugular vein for the 7 day old broilers, and a dosage of 0.35cc/kg was injected into the brachial vein of birds in other age groups. Directly post T-61 injection, the assigned euthanasia method was applied. Control birds received the T-61 injection, but no further euthanasia treatment. For the first 7 day old trial, and all 21 and 35 day old trials, each method was assigned to five birds, with an additional five birds serving as controls. After the initial use of the Zephyr at 7 days of age, the method was deemed unsuitable for aesthetic (excessive damage) and biosecurity reasons and its use at that age was not continued for the remainder of experiments. For the remainder of the 7 days trials five birds were used as control birds and the others were assigned either CD or KED. Throughout the experiments the euthanasia methods were performed by trained and experienced individuals. The cadavers were then transported to Prairie Diagnostics Services (Western College of Veterinary Medicine, University of Saskatchewan) for post-mortem data collection.

2.3.3.3 Live bird experiment

The Zephyr, CD and KED were evaluated on 240 live cull birds from five commercial farms at 7, 21 and 35 days of age. During the experiment each of the five farms was visited three times during the production cycle, and cull birds were provided from the same flock at 7, 21 and 35 days of age. During the data collection at 7 days of age, twelve cull birds were used and were assigned either manual or mechanical cervical dislocation. The data collections at 21 and 35 days of age tested 18 cull birds with six birds assigned to each euthanasia method. Birds were weighed and then the assigned euthanasia method was applied. Directly post euthanasia, the birds were assessed for onset of insensibility and death by measuring latency to behavioural and reflexive indicators. For unsuccessful euthanasia attempts, T-61 was used as a secondary euthanasia method. Once all on-farm data was collected, the bird cadavers were transported to Prairie Diagnostics Services, for further post-mortem data collection.

2.3.4 Data collection

2.3.4.1 Ante-mortem data collection – Indicators of insensibility and death

In the live experiment, immediately post euthanasia, a visual assessment was used to measure the latency to insensibility and death (Table 2.1). Birds were assessed for time until the absence of eye reflexes, pupillary light, palpebral blink and nictitating membrane reflex. In addition, they were monitored for latency to onset and cessation of involuntary behaviours including convulsions, cloacal winking and feather erection. Reflexes were monitored continuously, (in order of palpebral blink, nictitating membrane, pupillary light reflex and rhythmic breathing, followed by convulsions and cloacal winking) and were recorded from time of euthanasia to the onset or cessation of a reflex. Once absence of a reflex was noted, the reflex was reassessed to confirm absence and birds were monitored for possible return of a reflex. If a measure could not be assessed, it was marked as missing. Reflex assessments were performed by one trained assessor throughout the experiments for consistency. Palpebral blink reflex involved monitoring the blink or closing of the eyelids in response to the approach or touching of the cornea (Erasmus et al., 2010b). Pupillary light reflex was tested by monitoring for the constriction of the pupil in response to light being shone into the eye (Erasmus et al., 2010b), whilst the nictitating membrane reflex monitored the involuntary movement of the third eyelid, or nictitating membrane, in response to the approach or touching of the cornea and the medial canthus (Erasmus et al., 2010b). The onset of convulsions was considered as the point at which uncontrolled involuntary neuromuscular contractions occurred, whilst the end of convulsions was the point after both clonic (wing-flapping) and tonic (leg pedalling) convulsions ended, and the bird was motionless (Erasmus et al., 2010b). Cloacal winking was measured as the point at which the rhythmic opening and closing of the vent started and ended. Feather erection was the occurrence of sudden and global piloerection in insensible birds. When all reflexes, cloacal winking, convulsions or movement and breathing had terminated, the birds were considered dead. Birds were placed on their back and observed for righting reflex, none were seen throughout the experiment. Behaviours and what they are indicators of are further explained in Table 2.1.

Table 2. 1. Ethogram of reflexes and involuntary behaviours measured ante mortem after application of euthanasia method.

Measure	Type of Indicator	Meaning of indicator	Description
Pupillary light	Cranial nerve II/III	Midbrain dysfunction – reticular formation	Constriction of pupil in response to light being shone into eye
Palpebral blink	Cranial nerve V/VII	Midbrain dysfunction – reticular formation	Closing of eyelids (blinking) in response to approach or touching of cornea
Nictitating membrane	Cranial nerve V/IV	Midbrain dysfunction – reticular formation	Closure of the nictitating membrane in response to approach or touching of cornea and medial canthus
Cloacal winking	Cranial nerve X	Brainstem dysfunction	Opening and closing of vent
Feather erection	Cranial nerve X	Cardiac arrest	Global piloerection
Convulsions	Spinal cord effectors	Brainstem dysfunction and higher centre motor control	Uncontrolled involuntary muscle contractions Clonic – wing- flapping and leg pedaling Tonic – Muscle rigidity with final leg pedaling and wing-flapping

Adapted from Erasmus et al., 2010b; Martin et al., 2016; Terlouw et al., 2016b; Verhoeven et al., 2016.

2.3.4.2 Post-mortem data collection – Radiography

Four post mortem radiographs were taken of the cranial-cervical region of each bird. The radiographs were taken from the right and left lateral, dorsal and ventral views, using a Faxitron 43855D (Faxitron Bioptics, LLC, Tuscon, AZ, USA), in combination with CR 30-X digitiser (Agfa Healthcare NV, Mortsel, BE). The radiographs were evaluated using a RadiAnt Dicom Viewer 3.4.1 (Medixant, Poznan, Poland) for presence, location and distance of vertebrae dislocation, as well as the severity and number of fractures within vertebral column (Table 2.2 and Figure 2.2). Vertebrae dislocation was recorded by the presence of a separation between two vertebrae or between the atlas and the skull. The distance was recorded as a score based on distance between the vertebrae as a proportion to the length of the axis (C2) to account for bird size variation in the measurement.

Table 2. 2. Radiograph scoring scale for fracture severity and vertebra separation distance as a result of euthanasia method application.

Measure	Score	Severity descriptor
Fracture Severity		
	0	No fracture present
	1	Incomplete fracture
	2	Complete fracture
	3	Compound fracture
Separation distance		
	0	No separation of vertebrae
	1	Distance of separation is less than or half the length of axis
	2	Distance of separation is more than half or equal to the length of axis
	3	Distance of separation is more than the length of axis.

Woolcott et al., 2018a

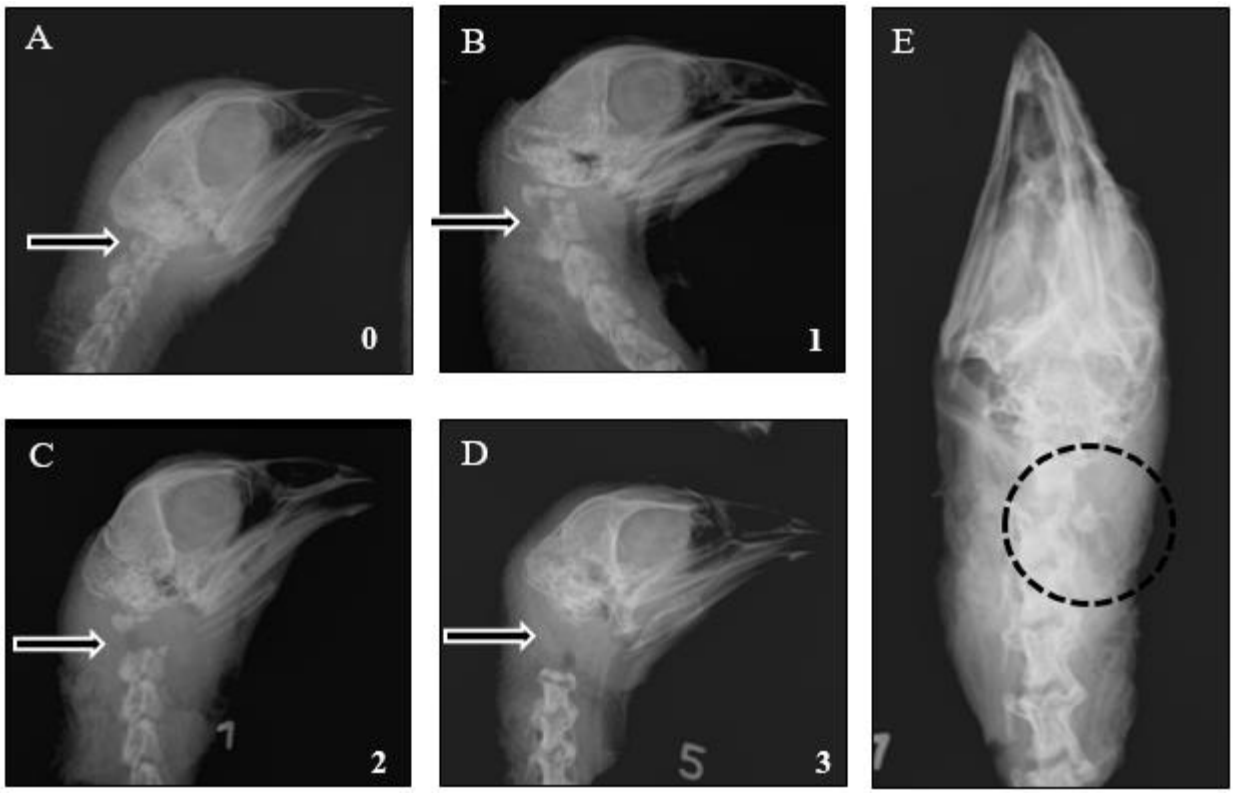

Figure 2. 2. Radiographs of the cranial-cervical region of broiler birds euthanized by cervical dislocation methods. A-D show the right lateral view and demonstrate the vertebrae separation distance. (A) No separation has occurred (Score = 0), (B) the distance of separation is less than or half the length of the axis [C2] (Score = 1), (C) the separation length is more than half the length of the axis or equal to the length of the axis (Score = 2), (D) the distance of separation is more than the length of the axis (Score = 3). Radiograph with a ventral view showing the presence of a (compound) fracture with multiple fracture pieces.

2.3.4.3 Post-mortem data collection – Gross Pathology

Post radiography, cadavers underwent gross anatomy evaluations of the cranial-cervical region and macroscopic scoring for lacerations of the skin, hemorrhaging, fracturing of the skull, and complete severing of the spinal cord. Macroscopic score scales developed for this procedure have been used in other euthanasia research (Table 2.3; Woolcott et al., 2018a). Skin laceration was assessed on a 0 to 2-point scale, and the assessment included laceration presence due to the euthanasia method and associated external blood loss (Figure 2.3). The skin was removed from the head, superior to the skull, and neck, and the tissues were assessed for subcutaneous hemorrhaging (extent of blood lost from vessels and pooled in surrounding tissues) (Figure 2.4).

The calvarium was assessed for extent of skull fracture, after which the calvarium, dura and brain were removed and subdural hemorrhaging was assessed on both the ventral and dorsal sides of the brain (Figure 2.5). Skull fracture was assessed on a 0 to 3-point scale, looking at complete fracturing and possible penetration (Figure 2.6). The vertebral column was then dissected to establish the presence of fractures within the vertebral column and ascertain complete severing of the spinal cord.

Table 2. 3. Macroscopic scoring scale for skin rupture, hemorrhage and skull fracture severity as a result of euthanasia method application.

Measure	**Score**	**Severity descriptor**
Skin Rupture		
	0	No laceration or skin break present
	1	Laceration or skin break with no external hemorrhage
	2	Laceration or skin break with external hemorrhage
Hemorrhage		
	0	No hemorrhage present
	1	<25% of area covered by hemorrhage
	2	25-50% of area covered by hemorrhage
	3	50-75% of area covered by hemorrhage
	4	>75% of area covered by hemorrhage
Skull fracture		
	0	No fracture present
	1	Depression/ incomplete fracture
	2	Complete fracture with no imbedded fragments
	3	Complete penetrating fracture with imbedded fragments

Woolcott et al., 2018a

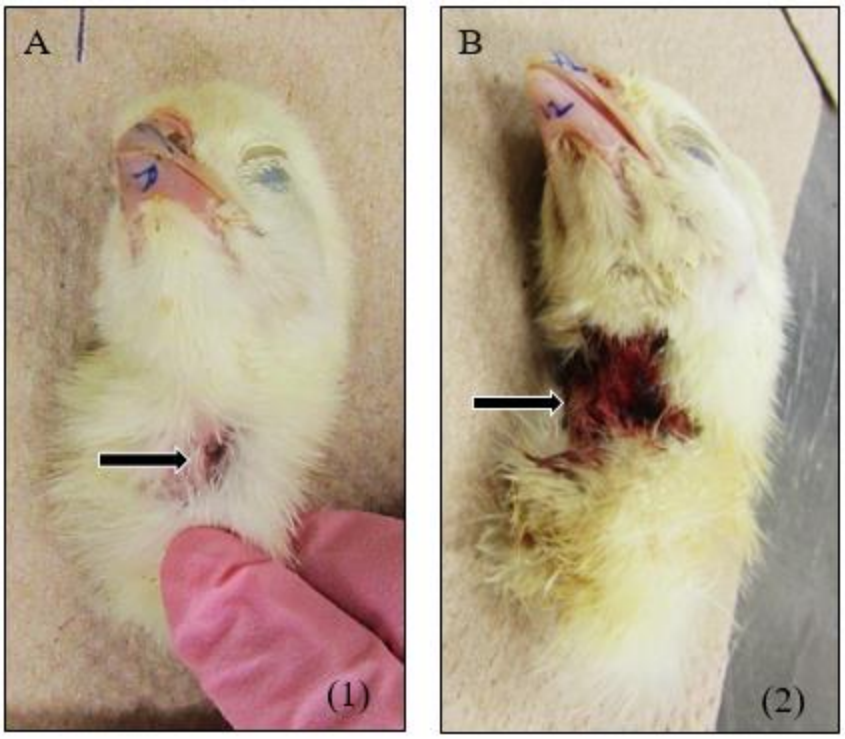

Figure 2. 3. Diagram showing examples of skin laceration from euthanasia with mechanical cervical dislocation. (A) A laceration on the neck without external hemorrhage (Score = 1) and (B) a neck laceration with external hemorrhage (Score = 2).

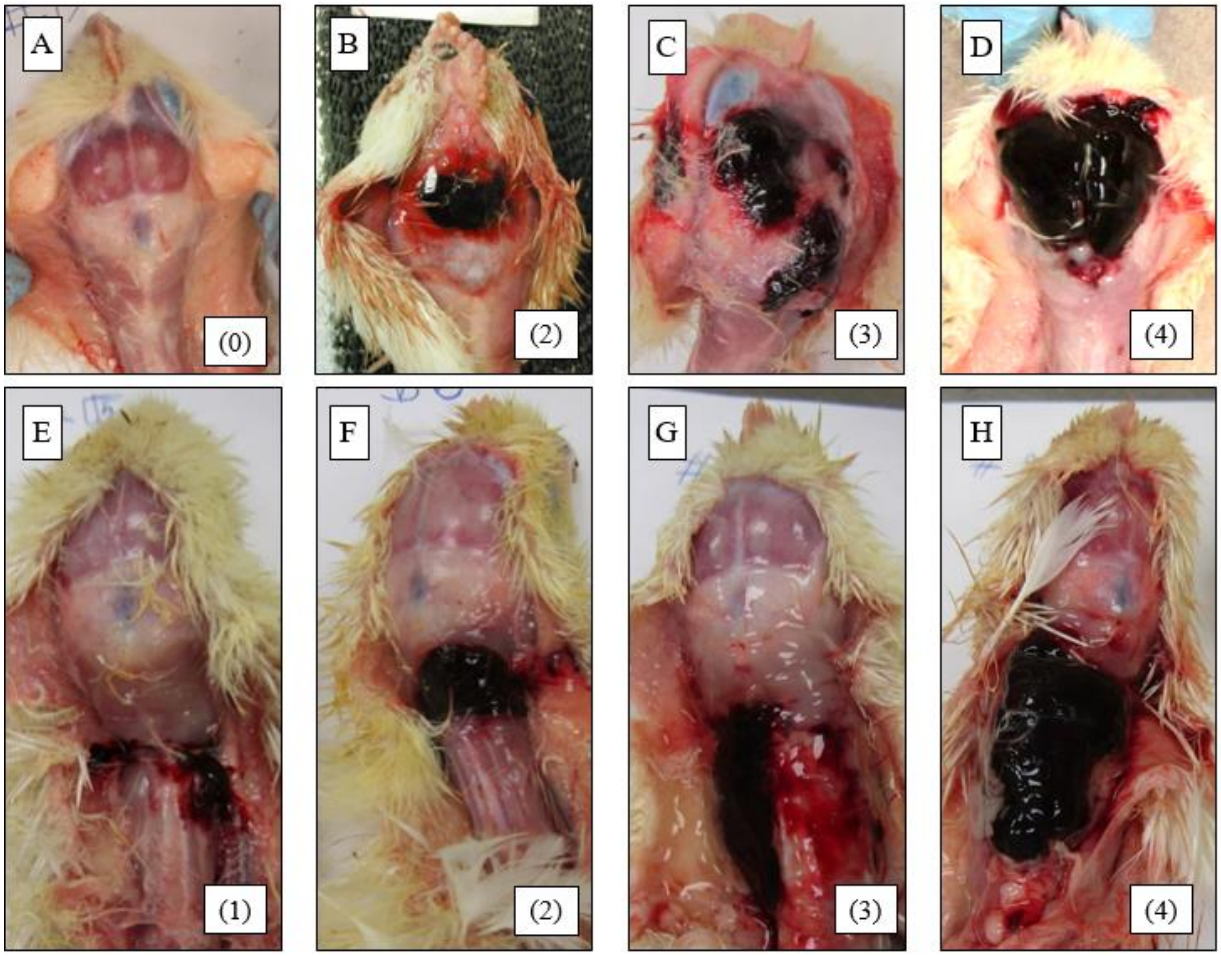

Figure 2. 4. Subcutaneous hemorrhaghing on the head and neck of broiler chickens as a result of on-farm euthanasia methods. A-D, subcutaneous hemorrhage scores for hemorrhages covering the dorsal surface of the head; (A) No hemorrrhage present on the head (Score = 0), (B) hemorrhaging covers between 25-50% of the head (Score = 2), (C) the subcutaneous hemorrhage covers 50-75% of the head (Score = 3), (D) over 75% of the head is covered by hemorrhage (Score = 4). E-F, illustrate subcutaneous hemorrhage scores for hemorrhaging on the dorsal and ventral sides of the neck; (E) less than 25% of the area is covered by hemorrhage (Score =1), (F) the hemorrhage covers between 25-50% of the neck (Score =2), (G) between 50-75% of the neck is covered by subcutaneous hemorrhage (Score =3), (H) the hemorrhage covers over 75% of the neck (Score = 4).

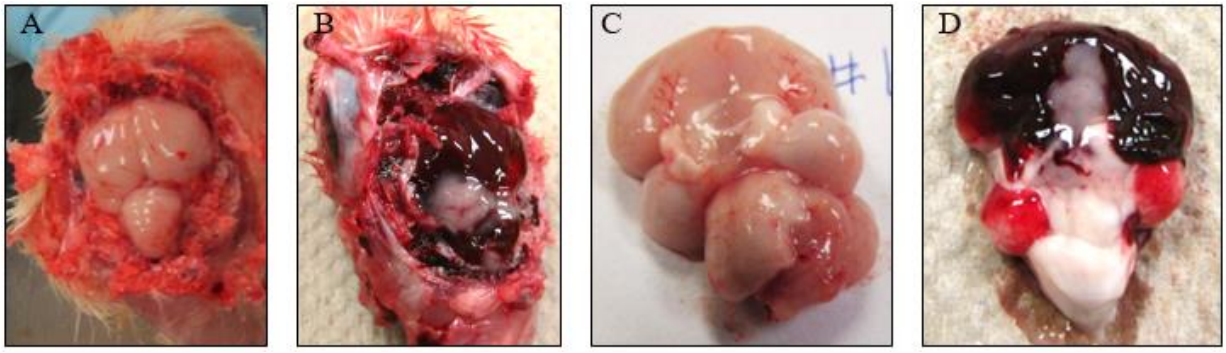

Figure 2. 5. Diagram with examples of subdural hemorrhaging on the dorsal and ventral sides of the brain. The dorsal side of the brain in situ, after removal of the calvarium and dura, with (A) and without (B) subdural hemorrhages. The ventral side of brain once removed from the skull, with no hemorrhaging (C) and significant subdural hemorrhaging (D).

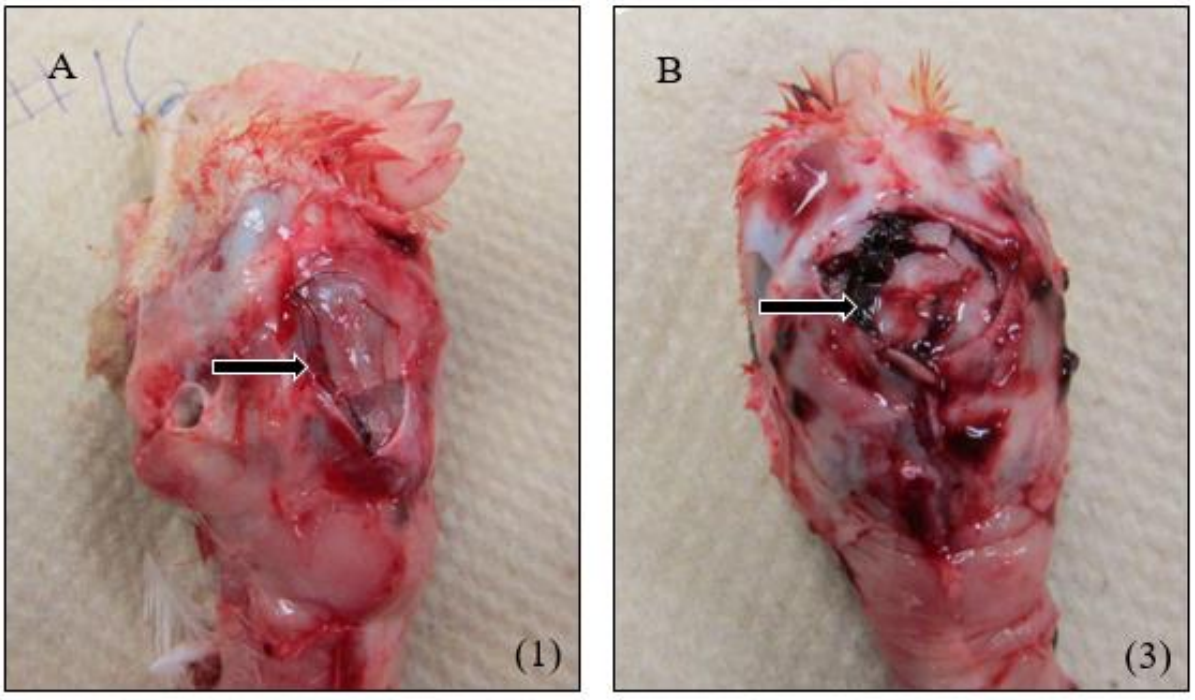

Figure 2. 6. Diagram showing examples of skull fractures from euthanasia with a non-penetrative captive bolt device. (A) An incomplete fracture of the skull (Score =1), and (B) a complete penetrating skull fracture with imbedded skull fragments (Score =3).

2.3.4.4 Post-mortem data collection – Histology

The brains, spinal cords and associated cranial-cervical tissues of a subsection of the birds (n= 50 (cadaver) and n= 80 (live)) were fixed in 10% buffered formalin for a minimum of 7 days in preparation for histological analyses. Once fixed, each brain was sectioned into three discrete regions; cerebral cortex, mid-brain and cerebellum, and brainstem. The spinal cord was also sectioned into three discrete regions in relation to atlas, axis and area post-severing site. These were sent to the University of Guelph, and macroscopic scoring of each section for subdural and parenchymal hemorrhages was performed by a veterinary pathologist (Dr. P.V. Turner). The score was based on the area of brain showing hemorrhage in relative proportion to total area of specimen on slide (Table 2.4; Woolcott et al., 2018a).

Table 2. 4. Histological scoring scale for subdural and parenchymal hemorrhage as a result of euthanasia method application.

Score	Severity	Proportion of specimen with hemorrhage
0	No hemorrhage	0
1	Minimal hemorrhage	<5%
2	Mild hemorrhage	5-10%
3	Moderate hemorrhage	>10-30%
4	Marked hemorrhage	>30%

Woolcott et al., 2018a

2.3.5 Statistical analyses

All statistical analyses were conducted using SAS 9.4 (SAS Inst. Inc., Cary, NC, USA). Data were (log+1) transformed for normality when required. The ante-mortem and post-mortem data were analysed as a randomised complete block design, with farm as block and euthanasia method as fixed effect, with bird as the experimental unit. Analyses of variance were performed on the ante mortem reflexes and behavioural indicators data with PROC MIXED, and means separations were conducted via the Tukey-Kramer method. The post-mortem results for radiography, gross anatomy and histology, were non-parametric ordinal score data and were ranked transformed via the Friedman transformation using PROC RANK. Treatment effects were investigated using the transformed data via the Friedman Test and via a one-way analysis of variance. The ANOVA was conducted using PROC MIXED, with means separation via the Tukey-Kramer method. Differences were considered significant when the probability of difference was less than 0.05, and trends were noted when less than or equal to 0.10.

2.4 Results

2.4.1 Cadaver post-mortem results

2.4.1.1 Radiography

At 7 days of age, the fracture severity of KED and CD birds did not differ from each other, but did differ from the controls (Table 2.5). Fracture severity differed as a result of euthanasia treatment at 21 and 35 days, with the severity of fractures being higher for KED than CD and control birds. At 7 days of age the fracture severity of KED and CD birds did not differ from each other but did differ from the controls. The scores for distance of separation at the site of dislocation was significantly greater for the CD birds than that of the KED birds at all ages.

Dislocation between skull and C1, C1 and C2 occurred more frequently with CD than KED, whilst the occurrence of dislocation between C2-C3 and C3-C4 was higher for KED than CD (Table 2.6) treatments. Dislocations occurring beyond C4 only occurred at 7 days of age for CD, and at 7 and 35 days of age for KED. Euthanasia attempts with incomplete dislocation only occurred with the KED device at 35 days of age.

Table 2. 5. Effect of cervical dislocation method on the post-mortem radiograph scores of fracture severity and separation distance on cadavers of broilers at 7, 21 and 35 days of age.

			Euthanasia Method				
Score	Age (d)	n	KED	CD	Control	*P* value	SEM[1]
Fracture severity[2]							
	7	55	1.7[a]	1.8[a]	0.0[b]	<0.01	0.17
	21	45	2.7[a]	1.0[b]	0.0[c]	<0.01	0.20
	35	45	2.0[a]	1.1[b]	0.0[c]	<0.01	0.15
Separation distance[3]							
	7	40	1.0[b]	2.3[a]	-	<0.01	0.17
	21	30	1.1[b]	2.8[a]	-	<0.01	0.19
	35	30	0.7[b]	2.9[a]	-	<0.01	0.22

[a-c] Rank means with common letter within a row do not differ significantly (P≤0.05). Real means are given in the table.

*KED = mechanical cervical dislocation, CD = manual cervical dislocation, Control = T-61 injection.

[1]Standard error of the mean.

[2]Scores for fracture severity: 0 = no fracture, 1 = incomplete fracture, 2 = complete fracture, 3 = compound fracture.

[3] Scores for separation distance: 0 = no separation 1 = ≤ ½ axis length, 2 = ≥ ½ axis length or = axis length, 3 = ≥ axis length.

Table 2. 6. The effect of cervical dislocation methods on the distribution of the location within the vertebral column at which the dislocation occurred for broilers at age 7, 21 and 35 days.

Age (d)	Treatment*	n	Skull-C1	C1-C2	C2-C3	C3-C4	C4+	No dislocation[1]
7	CD	19	1	0	6	7	5	-
	KED	19	1	2	11	4	1	-
21	CD	15	1	9	4	1	0	-
	KED	15	0	4	8	3	0	-
35	CD	15	4	7	4	0	0	-
	KED	15	0	0	4	7	2	2
Total	CD	49	6	16	14	8	5	0
	KED	49	1	6	23	14	3	2

*KED = mechanical cervical dislocation, CD = manual cervical dislocation.
[1] Spinal cord was not transected.

2.4.1.2 Gross pathology

The mean scores of subdural hemorrhaging, subcutaneous hemorrhaging on dorsal side of head and skull fractures were higher for Zephyr than the other euthanasia methods at 21 and 35 days of age (Table 2.7). At 7 days of age, a difference was also seen for subdural hemorrhaging with higher scores for CD then KED or control birds. The subcutaneous hemorrhage of the neck scores were higher for CD and KED than control birds at 7 days, and higher for CD and KED than both Zephyr and control broilers at 21 days. At 35 days, the neck hemorrhage score was highest for CD, then KED, and lowest for Zephyr and control. Scores for skin laceration were highest with the KED compared to Zephyr and control birds at 21 days of age with CD having intermediate scores. At 35 days of age, the KED resulted in higher skin laceration scores than all other treatments. Low subdural hemorrhaging scores were present in control birds at 7 days of age, but not at later ages. This hemorrhaging at 7 days of age resulted from the T-61 being injected into the jugular vine at this age, opposed to into the brachial vein as with the older birds. For 7 day old birds, CD and KED methods were found suitable for further investigation in experiment two, and for 21 and 35 day old birds the CD, KED and Zephyr were found suitable for further investigation.

Table 2. 7. Effect of euthanasia method on post-mortem gross pathology scoring for laceration, fracture and hemorrhaging on cadavers of broilers at 7, 21 and 35 days of age.

			Euthanasia Method*					
Region	Age (d)	n	KED	CD	Zephyr	Control	*P* value	SEM[1]
Subdural hemorrhaging[2]								
	7	55	0.0[b]	0.4[a]	-	0.1[b]	<0.01	0.05
	21	60	0.1[b]	0.2[b]	2.5[a]	0.1[b]	<0.01	0.15
	35	60	0.3[b]	0.3[b]	2.1[a]	0.1[b]	<0.01	0.13
Subcutaneous hemorrhaging on dorsal head[2]								
	7	55	0.0	0.0	-	0.0	0.40	0.02
	21	60	0.0[b]	0.0[b]	3.4[a]	0.0[b]	<0.01	0.20
	35	60	0.1[b]	0.0[b]	2.3[a]	0.0[b]	<0.01	0.16
Subcutaneous hemorrhaging on neck[2]								
	7	55	2.3[a]	2.5[a]	-	0.2[b]	<0.01	0.20
	21	60	2.8[a]	2.8[a]	0.0[b]	0.0[b]	<0.01	0.21
	35	60	2.4[b]	3.3[a]	0.3[c]	0.0[c]	<0.01	0.22
Skull fracture[3]								
	7	55	0.0	0.0	-	0.0	0.46	0.02
	21	60	0.0[b]	0.0[b]	2.9[a]	0.0[b]	<0.01	0.16
	35	60	0.0[b]	0.0[b]	2.7[a]	0.0[b]	<0.01	0.16
Skin laceration[4]								
	7	55	0.3	0.5	-	0.0	0.07	0.10
	21	60	0.4[a]	0.20[ab]	0.0[b]	0.0[b]	0.04	0.07
	35	60	0.7[a]	0.0[b]	0.0[b]	0.0[b]	<0.01	0.07

[a-c] Rank means with common letter within a row do not differ significantly (P≤0.05). Real means are given in the table.

*KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt, Control = T-61 injection.

[1]Standard error of the mean.

[2]Scores of proportion of tissue with hemorrhage: 0 = 0%, 1 = <25%, 2 = 25-50%, 3 = 50-75%, 4 = >75%.

[3]Scores for skull fracture: 0 = no fracture, 1 = depression/incomplete fracture, 2 = complete fracture with no imbedded fragments, 3 = complete penetrating fracture with imbedded fragments.

[4]Scores for skin laceration: 0 = no laceration, 1 = laceration with no external hemorrhage, 2 = laceration with external hemorrhage.

2.4.1.3 Histology

The results indicated that for birds at 7 days of age, a difference in the effect of euthanasia treatments on hemorrhaging of the brain was only seen for parenchymal hemorrhages in the spinal cord, with higher scores for KED than CD (Table 2.8). A trend towards a difference for subdural hemorrhages in the spinal cord was also noted. At 21 days of age, a difference due to treatment was found for parenchymal hemorrhage in the midbrain, with scores higher for Zephyr than CD or KED. A similar effect was seen for subdural hemorrhaging, with hemorrhage scores being higher with use of the Zephyr than both the CD and KED, in the pallium, midbrain and hindbrain. Birds at 35 days of age showed higher subdural hemorrhaging scores for Zephyr than KED and CD, in the pallium and midbrain, and higher scores for Zephyr birds than KED in the hindbrain.

Table 2. 8. Effect of euthanasia method on parenchymal and subdural hemorrhage scores in the pallium, midbrain, hindbrain and spinal cord of a subsample of broiler cadavers at 7 (n=12), 21 (n=18) and 35 (n=18) days old.

			Euthanasia Method (score)[1]				
Age (d)	Hemorrhage type	Brain Region	KED	CD	Zephyr	*P* value	SEM[2]
7	Parenchymal	Pallium	0.0	0.0	-	--	0.00
		Midbrain	0.2	0.0	-	0.33	0.08
		Hindbrain	0.0	0.0	-	--	0.00
		Spinal cord	0.8[a]	0.0[b]	-	0.04	0.23
	Subdural	Pallium	0.0	0.0	-	--	0.00
		Midbrain	0.5	0.2	-	0.47	0.19
		Hindbrain	0.3	0.0	-	0.34	0.17
		Spinal cord	1.3	0.2	-	0.06	0.33
21	Parenchymal	Pallium	0.0	0.0	0.5	0.16	0.13
		Midbrain	0.0[b]	0.0[b]	1.2[a]	<0.01	0.20
		Hindbrain	0.0	0.0	0.2	0.35	0.05
		Spinal cord	1.2	0.7	0.2	0.64	0.27
	Subdural	Pallium	0.0[b]	0.0[b]	3.0[a]	<0.01	0.42
		Midbrain	0.0[b]	0.0[b]	2.7[a]	<0.01	0.41
		Hindbrain	0.0[b]	0.7[b]	3.5[a]	<0.01	0.34
		Spinal cord	1.3	1.2	1.7	0.73	0.33
35	Parenchymal	Pallium	0.0	0.0	0.0	--	0.00
		Midbrain	0.0	0.0	0.0	--	0.00
		Hindbrain	0.0	0.0	0.0	--	0.00
		Spinal cord	1.2	1.0	-	0.63	0.31
	Subdural	Pallium	0.0[b]	0.3[b]	2.3[a]	<0.01	0.34
		Midbrain	0.2[b]	0.2[b]	3.0[a]	<0.01	0.38
		Hindbrain	1.0[b]	1.8[ab]	3.3[a]	0.02	0.35
		Spinal cord	1.2[b]	2.7[a]	-	0.02	0.35

[a,b] Rank means with common letter within a row do not differ significantly (P≤0.05). Real means are given in the table.

[1]Scores of proportion of tissue with hemorrhage on 4-point scale: 0= 0%, 1 = <5%, 2 = 5-10%, 3 = >10-30%, 4 = >30%.

[2]Standard error of the mean.

- Zephyr not used at 7 days of age.

-- No *P* value as convergence criteria could not be met as there was no difference between scores.

2.4.2 Live bird study ante mortem results

Pupillary light reflex and palpebral blink reflex demonstrated a difference between CD and KED (Table 2.9). The latency to loss of pupillary light, palpebral blink and nictitating membrane was the shortest with the Zephyr, then CD and longest with KED, at 21 and 35 days of age.

Euthanasia methodology resulted in a difference in latency to feather erection at 21 and 35 days of age, with the latency being longer with the use of KED than either Zephyr or CD (Table 2.10). Rhythmic breathing ended sooner with use of the Zephyr, compared to the other two methods. Bird latency to cloacal winking demonstrated a difference between treatments, with the shortest latency found with use of the CD at all ages. An effect of euthanasia method was found on convulsions. Both the latency to cessation of convulsions and the duration of convulsions were shortest with use of CD when compared to the KED at 7 days and compared to KED and Zephyr at 21 days. At 35 days the latency to cessation of convulsions was shortest for CD compared to Zephyr, with a trend towards CD resulting in the shortest duration.

Table 2. 9. Effect of euthanasia method on time (s) from euthanasia method application to loss of pupillary light, palpebral blink and nictitating membrane reflexes in broiler chickens at 7, 21 and 35 days of age.

			Euthanasia Method*				
Reflex (s)	Age (d)	n	KED	CD	Zephyr	*P* value	SEM[1]
Pupillary light	7	60	57.87[a]	30.50[b]	-	<0.01	4.403
	21	91	64.43[a]	37.97[b]	1.01[c]	<0.01	2.703
	35	90	76.07[a]	46.13[b]	1.80[c]	<0.01	2.663
Palpebral blink	7	60	23.07[b]	29.66[a]	-	0.04	2.201
	21	91	21.13[a]	16.23[b]	1.09[c]	<0.01	1.543
	35	90	26.20[a]	14.53[b]	1.80[c]	<0.01	1.250
Nictitating membrane	7	60	36.03	30.70	-	0.34	3.906
	21	91	37.53[a]	22.73[b]	1.18[c]	<0.01	2.308
	35	90	68.30[a]	25.30[b]	1.80[c]	<0.01	2.992

[a-c] Means with common letter within a row do not differ significantly ($P \leq 0.05$).
*KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt.
[1]Standard error of the mean.

Table 2. 10. Effect of euthanasia method on the onset of feather erection, the cessation of rhythmic breathing and cloacal winking and the start, the end time and overall length of convulsions (s) in broiler chickens at 7, 21 and 35 days of age.

			Euthanasia Method*				
Reflex (s)	Age (d)	n	KED	CD	Zephyr	*P* value	SEM[1]
Feather erection							
Onset	7	60	135.17	93.17	-	0.63	43.766
	21	87	82.88[a]	61.54[b]	64.29[b]	<0.01	4.056
	35	86	82.41[a]	67.49[b]	59.20[b]	<0.01	3.358
Rhythmic breathing							
Cessation	7	51	109.28	51.60	-	0.05	16.442
	21	91	50.33[a]	39.30[a]	19.94[b]	<0.01	4.000
	35	89	12.73[a]	12.17[a]	1.80[b]	<0.01	1.214
Cloacal Winking							
Cessation	7	55	128.21[a]	86.33[b]	-	<0.01	7.897
	21	88	120.42[a]	89.87[b]	117.33[a]	<0.01	6.274
	35	88	124.43[a]	101.77[b]	126.13[a]	0.01	6.022
Convulsions							
Onset	7	60	1.50	5.30	-	0.34	2.815
	21	91	1.47	1.00	7.60	0.44	4.103
	35	90	1.13	1.00	1.80	0.44	0.469
Cessation	7	60	147.43[a]	77.90[b]	-	<0.01	11.528
	21	91	112.07[a]	79.53[b]	104.05[a]	<0.01	6.569
	35	90	114.97[ab]	93.23[b]	124.17[a]	0.05	9.089
Duration	7	60	145.93[a]	72.60[b]	-	<0.01	11.344
	21	91	110.60[a]	78.53[b]	102.14[a]	<0.01	6.734
	35	90	113.83	92.23	122.37	0.06	9.086

[a,b] Means with common letter within a row do not differ significantly (P≤0.05).
*KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt, Control = T-61 injection.
[1]Standard error of the mean.

2.4.3 Live bird study post-mortem results

2.4.3.1 Radiography

Regardless of age, more severe fractures were noted with the use of the KED than with CD. The distance of separation was consistent between the ages, with the distance of separation at the dislocation site larger for the CD birds then the KED birds (Table 2.11).

A difference was seen in number of fractures within the vertebral column at 21 and 35 days of age, with a higher number of fracture pieces noted when the KED device was used. The range of fracture pieces was also larger for the KED then for CD (Table 2.12).

With the use of either CD or KED, the dislocation below C3 only occurred at 7 days of age, with the dislocations getting closer to the skull as the birds age (Table 2.13). Overall, the most dislocations occurring between the skull and C1 were noted with the use of CD. Unsuccessful dislocations were only noted with the use of the KED, with 5.5% of attempts having the spinal cord still attached.

Table 2. 11. Effect of cervical dislocation method on the post-mortem radiograph scores of fracture severity and separation distance on broilers at 7, 21 and 35 days of age.

			Euthanasia Method			
Score	Age (d)	n	KED	CD	*P* value	SEM[1]
Fracture severity[2]						
	7	60	1.9[a]	1.2[b]	0.02	0.16
	21	60	2.0[a]	1.1[b]	<0.01	0.16
	35	60	1.8[a]	1.0[b]	<0.01	0.14
Separation distance[3]						
	7	60	1.0[b]	3.0[a]	<0.01	0.14
	21	60	1.4[b]	3.0[a]	<0.01	0.12
	35	60	1.3[b]	3.0[a]	<0.01	0.12

[a,b] Rank means with common letter within a row do not differ significantly (P≤0.05). Real means are given in the table.
*KED = mechanical cervical dislocation, CD = manual cervical dislocation.
[1]Standard error of the mean.
[2]Scores for fracture severity: 0 = no fracture, 1 = incomplete fracture, 2 = complete fracture, 3 = compound fracture.
[3] Scores for separation distance: 0 = no separation 1 = ≤ ½ axis length, 2 = ≥ ½ axis length or = axis length, 3 = ≥ axis length.

Table 2. 12. Effect of euthanasia method on the number of fractures of the vertebral column.

Age		Euthanasia Method*										
(d)	n	KED			CD			Zephyr			*P* value	SEM[1]
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max		
7	60	1.5	0	5	1.0	0	4	-	-	-	0.24	0.26
21	91	1.1[a]	0	5	0.5[b]	0	4	0[c]	0	0	<0.01	0.14
35	90	1.8[a]	0	3	0.8[b]	0	3	0[c]	0	0	<0.01	0.21

[a,b] Means with common letter within a row do not differ significantly (P≤0.05).
*KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt.
[1]Standard error of the mean.

Table 2. 13. The distribution of the location within the vertebral column at which the dislocation occurred for two cervical dislocation methods performed on broilers at 7, 21 and 35 days of age.

Age (d)	Treatment	n	Skull-C1	C1-C2	C2-C3	C3-C4	C4+	No dislocation[1]
7	CD	30	0	0	1	11	18	-
	KED	30	2	11	9	1	3	4
21	CD	30	14	16	0	0	0	-
	KED	30	0	9	20	0	0	1
35	CD	30	19	11	0	0	0	-
	KED	30	2	20	7	0	0	1
Total	CD	90	33	27	1	11	18	0
	KED	90	4	40	36	1	3	6

[1] Spinal cord was not transected.

2.4.3.2 Gross pathology

Subdural hemorrhaging scores differed by treatment at 21 and 35 days, with Zephyr resulting in the highest score, followed by CD and then KED (Table 2.14). Subcutaneous hemorrhaging of the head and skull fracture also showed a treatment effect, with the highest scores occurring with use of the Zephyr. At 7 days of age the scores for subcutaneous hemorrhage of the neck were higher for birds euthanized with CD than bird euthanized with KED, and at 21 and 35 days the score resulting from the KED and CD were higher than those for Zephyr. A treatment effect was seen for skin laceration scores, with CD resulting in scores differing from the KED at 21 days, and the CD scores differing from both the Zephyr and KED at 35 days.

The overall percentage of complete transections of the spinal cord for CD was 98%, whilst that of KED was 81% (Table 2.15). The higher percentage of complete transections with CD compared to KED was mirrored by the percentages of complete transections at days 7 and 35.

Table 2. 14. Effect of euthanasia method on post-mortem gross pathology scores for laceration, fracture and hemorrhaging in broilers at 7, 21 and 35 days of age.

			Euthanasia Method*				
Region	Age	n	KED	CD	Zephyr	*P* value	SEM[1]
Subdural hemorrhaging[2]							
	7	60	0.1	0.0	-	0.55	0.03
	21	91	0.1[c]	0.5[b]	3.2[a]	<0.01	0.16
	35	90	0.2[c]	0.5[b]	3.0[a]	<0.01	0.15
Subcutaneous hemorrhaging on dorsal head[2]							
	7	60	0.1	0.1	-	0.62	0.034
	21	91	0.1[b]	0.1[b]	3.8[a]	<0.01	0.19
	35	90	0.7[b]	0.1[c]	3.8[a]	<0.01	0.20
Subcutaneous hemorrhaging on neck[2]							
	7	60	1.5[b]	3.5[a]	-	<0.01	0.18
	21	91	3.8[a]	3.9[a]	1.0[b]	<0.01	0.18
	35	90	3.5[a]	3.7[a]	1.0[b]	<0.01	0.18
Skull fracture[3]							
	7	60	0.0	0.0	-	-	0.00
	21	91	0.0[b]	0.1[b]	2.8[a]	<0.01	0.14
	35	90	0.0[b]	0.0[b]	2.7[a]	<0.01	0.14
Skin laceration[4]							
	7	60	0.7	0.6	-	0.77	0.12
	21	91	0.9[a]	0.1[b]	0.4[ab]	<0.01	0.09
	35	90	1.0[a]	0.1[b]	1.3[a]	<0.01	0.10

[a,b] Rank means with common letter within a row do not differ significantly (P≤0.05). Real means are given in the table.

*KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt.

[1]Standard error of the mean.

[2]Scores of proportion of tissue with hemorrhage: 0 = 0%, 1 = <25%, 2 = 25-50%, 3 = 50-75%, 4 = >75%.

[3]Scores for skull fracture: 0 = no fracture, 1 = depression/incomplete fracture, 2 = complete fracture with no imbedded fragments, 3 = complete penetrating fracture with imbedded fragments.

[4]Scores for skin laceration: 0 = no laceration, 1 = laceration with no external hemorrhage, 2 = laceration with external hemorrhage.

Table 2. 15. Effect of euthanasia method on percentage of complete spinal cord transection for broilers at 7, 21, and 35 days of age.

Age (d)	Euthanasia Method		
	KED	CD	ZEPHYR
7	53	100	-
21	100	97	-
35	90	100	-
Total	81	98	-

2.4.3.3 Histology

At seven days of age, a difference in hemorrhaging of the brain was only seen for parenchymal hemorrhaging of the spinal cord, with KED resulting in higher hemorrhage scores (Table 2.16). Differences were noted in more variables as the birds aged, with differences between treatments for both parenchymal and subdural hemorrhaging throughout the brain regions found at both 22 and 35 days. For both hemorrhage types and at both ages, the hemorrhage scores with use of the Zephyr were higher than both CD and KED in both the cortex and the midbrain. Spinal cord hemorrhaging differed between the methods at 21 days of age, with parenchymal hemorrhaging being higher for KED than Zephyr and subdural hemorrhage scores being higher for both KED and CD compared to Zephyr. Differences in hemorrhaging were also seen in the hindbrain at both 21 and 35 days, with 21 days showing a difference in subdural hemorrhage scores and 35 days showing a difference for both parenchymal and subdural hemorrhage scores, with the lowest scores being seen when the KED device was used.

Table 2. 16. Effect of euthanasia method on parenchymal and subdural hemorrhage scores in the pallium, midbrain, hindbrain and spinal cord of a subsample of broilers at 7 (n=20), 21 (n=30) and 35 (n=30) days of age.

Age (d)	Hemorrhage type	Brain Region	Euthanasia Method (score)[1]				
			KED	CD	Zephyr	*P* value	SEM[2]
7	Parenchymal	Pallium	0.0	0.0	-	--	0.00
		Midbrain	0.0	0.0	-	--	0.00
		Hindbrain	0.2	0.0	-	0.23	0.10
		Spinal cord	1.8[a]	0.4[b]	-	0.02	0.28
	Subdural	Pallium	0.1	0.0	-	0.33	0.05
		Midbrain	0.0	0.0	-	--	0.00
		Hindbrain	0.7	0.2	-	0.48	0.22
		Spinal cord	0.9	0.6	-	0.44	0.23
21	Parenchymal	Pallium	0.0[b]	0.0[b]	1.1[a]	<0.01	0.16
		Midbrain	0.0[b]	0.0[b]	1.1[a]	<0.01	0.15
		Hindbrain	0.0	0.1	0.5	0.07	0.09
		Spinal cord	1.5[a]	0.4[ab]	0.0[b]	0.02	0.21
	Subdural	Pallium	0.0[b]	0.0[b]	3.8[a]	<0.01	0.33
		Midbrain	0.1[b]	0.4[b]	3.6[a]	<0.01	0.32
		Hindbrain	0.0[c]	1.8[b]	3.2[a]	<0.01	0.28
		Spinal cord	1.7[a]	2.1[a]	0.0[b]	<0.01	0.26
35	Parenchymal	Pallium	0.0[b]	0.0[b]	1.3[a]	<0.01	0.16
		Midbrain	0.0[b]	0.2[b]	1.4[a]	0.02	0.17
		Hindbrain	0.0[b]	0.2[ab]	0.7[a]	<0.01	0.12
		Spinal cord	1.2	0.3	1.0	0.08	0.21
	Subdural	Pallium	0.0[b]	0.0[b]	2.6[a]	<0.01	0.27
		Midbrain	0.0[b]	0.6[b]	2.6[a]	<0.01	0.29
		Hindbrain	0.8[b]	2.2[a]	3.1[a]	<0.01	0.27
		Spinal cord	1.1	2.1	1.2	0.25	0.28

[a,b] Rank means with common letter within a row do not differ significantly (P≤0.05). Real means are given in the table.
[1]Scores of proportion of tissue with hemorrhage on 4-point scale: 0= 0%, 1 = <5%, 2 = 5-10%, 3 = >10-30%, 4 = >30%.
[2]Standard error of the mean.
- Zephyr not used at 7 days of age.
-- No *P* value as convergence criteria could not be met as there was no difference between scores.

2.4.4 Success rate

The success rate, for death resulting from first application of treatment method, was 100% for CD, 100% for KED and 98% for Zephyr. During the experiment there was one incidence of the Zephyr misfiring; this occurred at 35 days of age and a second application of the Zephyr occurred resulting in a successful onset of death.

2.5 Discussion

For euthanasia methods to be efficacious for on-farm use and have a positive impact on bird welfare/humaneness, they need to be reliable, render the bird insensible and dead rapidly, and result in these without unnecessary trauma or tissue damage that may result in additional pain or distress. This study investigated three commercially available euthanasia methods for use on farms, to evaluate each for their efficacy at three ages representative of three stages of the broiler production cycle: brooding, growing and finishing phases. Throughout the production cycle, birds need to be culled for humane reasons and the efficacy of the methods for the euthanizing of birds may vary with the different ages and body conformation as they develop.

2.5.1 Traumatic injury and physical damage

2.5.1.1 Non-penetrative captive bolt

The post-mortem scores are indirect measures of traumatic brain injury, which is desirable for euthanasia methods that use concussive force. The extent of the scores noted with use of the Zephyr are evidence that the Zephyr has sufficient concussive force to disrupt brain function and cause insensibility and brain death for broilers. The severity of skull fractures is indicative of brain damage severity (Erasmus et al., 2010c; Tseng et al., 2011; Casey-Trott et al., 2013; Woolcott et al., 2018a), and is associated with higher incidence of mortality (Tseng et al., 2011). Similar to previous studies with turkeys, our research showed a high severity of skull fracture with Zephyr use, including a high number of skull fractures involving the displacement of skull fragments into the underlying neural tissues (Erasmus et al., 2010c; Woolcott et al., 2018a). These displaced fractures often penetrated into the neural tissue, causing further physical damage to the brain. The high incidence of severe skull fractures and the penetration of skull fragments indicate the tissue damage resulting from the Zephyr would be sufficient to cause

physical and functional impairment of the brain, resulting in insensibility and death (Tseng et al., 2011).

Macroscopic scores for subcutaneous hemorrhaging on the head and subdural hemorrhaging were severe when the Zephyr was used at all ages tested, an outcome previously reported in various other animal species (Erasmus et al., 2010c [turkeys]; Casey-Trott et al., 2013, 2014 [pigs]; Woolcott et al., 2018a [turkeys]). The high scores for these hemorrhages indicate that the concussive force of the bolt results in contusions and hemorrhaging to both the tissue of the head and brain (Erasmus et al., 2010c; Woolcott et al., 2018a), signifying damage to the neural tissue and vasculature. Hemorrhaging occurred on both the dorsal side (coup) and ventral side of the brain (counter-coup), confirming that the force of the bolt impact caused tissue damage, both from deformation of the skull and the bolt impact as well as from the collision of the brain with the opposite side of the cranium (Shaw, 2002; Gaetz, 2004; Andriessen et al., 2010; Martin, 2015; Grist et al., 2017). Furthermore, the presence of the hemorrhages suggest that the severity of blood lost from the brain vasculature into the brain tissue and cranial fault would lead to secondary injuries, such as increased intracranial pressure and hypoxic-ischemia (Gaetz, 2004; Pearce, 2008; Andriessen et al., 2010; Xiong et al., 2013).

Moderate to high histological scores were found for both subdural and parenchymal hemorrhaging in all three regions of the brain; pallium, midbrain and hindbrain. These are clear indicators of brain damage, as the presence of contusions, vascular damage and hemorrhaging indicate a large volume of blood was lost from the vascular system and infiltrated the brain (Woolcott, 2007; Woolcott et al., 2018a) in these regions. The extensive damage to all of these regions means the device successfully affected the structure of these regions and rendered them non-functional (Casey-Trott et al., 2013; Woolcott et al., 2018a), thus successfully inhibiting consciousness, as well as the motor and sensory systems, cognition, respiration and reflexes (Martin, 2015). Our results mirror those from a study investigating the Zephyr for the euthanasia of turkeys, which showed that the extent and severity of damage caused by the Zephyr is substantial to the deep brain regions that are targeted and control sensibility and vital functions (Woolcott et al., 2018a).

2.5.1.2 Cervical dislocation

Cervical dislocation requires both a severing of the spinal column and a rupturing of the carotid arteries to ensure the onset of cerebral ischemia and disrupt the transfer of sensory and motor information between the brain and body. Both the CD and KED were successful at inducing death and resulted in spinal cord transection (complete or partial), with complete transections percentages of 81% and 98% for KED and CD, respectively across all ages. High subcutaneous hemorrhages scores on the neck were also found for both methods indicating the successful rupture of one or both carotid arteries. The successful rupturing of one or more of the carotid arteries means the methods reduced the blood flow to the brain and arterial pressure leading to cerebral ischemia and hypoxia.

Previous research into impacts of CD noted subdural hemorrhage with CD and attributed this to the extensive blood loss from the rupturing of the carotid arteries (Erasmus et al., 2010c). Our research also found subdural hemorrhaging, as well as parenchymal hemorrhaging, in the hindbrain as a result of CD. Rather than just being a pooling of blood from the carotid arteries, these hemorrhages could also indicate that CD has a concussive effect on the brain, as previously suggested by Gregory and Wotton (1990). The combined presence of both hemorrhage types is similar to the hemorrhages ascribed to brain tissue damage from concussive trauma, such as that of blunt force trauma or NPCD. Furthermore, the lack of such hemorrhages seen for the KED devices, even though the subcutaneous hemorrhage neck scores were equivalent, suggests that in our studies with broilers, the explanation for these scores may go beyond pooling of blood from rupturing of carotid arteries. A dislocation at C0-C1 is preferred, as it is believed to increase the likelihood of the dislocation also having a concussive effect on the brainstem or top of the spinal cord (Gregory and Wotton, 1990; Sparrey et al, 2014; Martin et al., 2016). Our research showed that with the exception of the birds at day 7, the highest percentage of dislocation at C0-C1 resulted from the CD as compared to the KED. This combined with the presence of hemorrhaging indicative of the concussion and the shorter time to insensibility and death, when compared to the KED, suggests that the dislocation at C0-C1 could indeed be beneficial for good cervical dislocation and concussive effects resulting in insensibility. However, although a dislocation that occurs further down the vertebral column, where just a severing of the spinal

cord and a rupturing of the arteries occurs, is sufficient to result in death, it will increase the time to insensibility (Martin, 2015).

Previous research studying various mechanical cervical dislocation devices, such as the Burdizzo or Semark pliers, have shown these to be inappropriate as they result in crushing rather than a twist and stretch of the neck (Gregory and Wotton, 1990; Sparrey et al., 2014; Martin et al., 2016). The crushing injuries do not cause cerebral ischemia, but rather cause death by asphyxia and often also result in excessive and possibly painful damage (Gregory and Wotton, 1990; Sparrey et al., 2014; Martin et al., 2016). The KED was reported to be designed as a purpose-built mechanical cervical device that uses shearing and twisting instead of crushing (Koechner et al., 2012). In line with previous findings for broilers of 36, 42 and 43 days of age (Jacobs et al., 2019), the device did result in a separation distance between the vertebrae, but one that was significantly shorter than that seen with CD, which indicated the device may not result in enough or as much stretching force as intended. Furthermore the higher number and severity of fractures within the spinal column seen with the KED are an issue as the presence of fractures, resulting from a cervical dislocation method, may be painful, although no data specific to this fracture type appears to be available to confirm this (AVMA, 2013; NFACC, 2016a). The presence of these fractures and the shorter separation distance suggest the KED is severing the spinal cord by forcing apart vertebrae rather than a mechanical twisting and stretching.

It has been hypothesised by Martin (2015) that assisted CD, in the use of the pail edge for young birds, does not have the same effects as normal CD as there is not sufficient stretch and tear motion. The results for CD used with birds in the starting phase from our study indicate that this could be the case, as although the person performing the euthanasia was a trained stock person, the dislocations were further down the spinal cord and more fractures of the vertebrae were noted than when CD was used at other ages.

2.5.1.3 Skin lacerations

Skin lacerations, with and without blood loss were seen with use of both the Zephyr and the KED. Recent research investigating CD and KED for euthanasia of broilers at 36, 42 and 43 days of age, also found the KED to result in skin lacerations with external blood loss occurring with 75-95% of the euthanasia attempted (Jacobs et al., 2019). These numbers are higher than

those in our study, with KED resulting in skin laceration 47% of the time, and Zephyr 42% of the time. Although the amount of blood lost was not quantified, there were occasional incidences with a high volume of blood loss but with the majority of cases the blood loss was minimal. Although the presence of skin lacerations and blood loss does not necessarily affect the efficacy of the device, it is important to consider the potential impacts of the blood as a biohazard or biosecurity risk (Galvin et al., 2005), as birds culled may contain blood-borne infectious agents or other hazards for the rest of the flock. Woolcott and colleagues (2018a) suggested that in order to minimise the possibility of blood loss or external hemorrhaging with the Zephyr, it may be an option to alter the force of the bolt by altering the compressor pressure. This was not an option with our particular Zephyr setup as it used the corresponding system driven by pressurized CO_2, which does not allow for an adaptation of the pressure. Furthermore, the altering of the compressor pressure and thus bolt force, if untested, may result in an increase in misfires or unsuccessful euthanasia attempts.

2.5.2 Insensibility and death

The ante-mortem behavioural indicators showed the shortest latency to insensibility when the Zephyr was used (shortest latencies to loss of brain stem reflexes) whilst the shortest time to death occurred with the use of CD. The almost instantaneous loss of brain stem reflexes seen with the Zephyr (< 2 s) is in line with previous findings with another NPCD; the Turkey Euthanasia Device resulted in a loss of nictitating membrane reflex within 2 s of applying the technique when euthanizing broilers of 3.5 kg (Hulet et al., 2013). There is a suggestion in the literature that the impact of the bolt might alter the response of eye reflexes (Martin et al., 2016; Terlouw et al., 2016b), thus the eye reflexes may not be accurate for reporting time of loss of consciousness. However, the birds in this experiment also showed a rapid or immediate loss of rhythmic breathing, which is an indicator that the respiratory centres in the brainstem have been rendered dysfunctional, and that the bird is unconscious and possibly brain dead (Terlouw et al., 2016b). Thus, the cessation of both the brain reflexes and rhythmic breathing occurred almost directly after the application of the Zephyr suggests that the Zephyr results in a rapid loss of consciousness. The corneal or palpebral blink reflex as well as the nictitating membrane reflex, have both been suggested to be indicators of brain death, rather than solely indicators of insensibility (Sandercock et al., 2014; Martin et al., 2016). However, these studies investigated the indicators on anaesthetised birds, with anesthesia having the potential to affect brainstem

reflexes (Martin, 2015). The three eye reflexes were chosen for this experiment, as they are conservative indicators of insensibility, meaning that the absence of these indicators shows that consciousness is absent but their presence does not necessarily indicate consciousness. Thus, whether these indicators are signals of brain death or of insensibility, their absence indicates the bird is no longer able to receive or integrate sensory information, thus is not able to experience pain or distress. Measuring the absence of these indicators is a good tool to measure the latency to the time in which the bird is not conscious to the euthanasia process and any welfare impacts associated.

Manual cervical dislocation resulted in the shortest time to the end of cessation of convulsions and cloacal winking, and to total cessation of movement. Cessation of movement is often used in euthanasia, depopulation and slaughter research as an indicator of death (Gerritzen et al., 2004; Dawson et al., 2007; Dawson et al., 2009). As CD also had the shortest latencies to feather erection, an indicator of cardiac arrest, CD has the shortest time to cessation of cardiac function as well as brain death. The short time to cardiac arrest and total cessation of movement suggests that the combined effects of cerebral ischemia, disruptive damage to the neural tissues of the spinal cord, and the concussion damage to the brainstem, have a combinatory effect on causing the ending of life. This combination of effects with CD is more efficient than when just cerebral ischemia and spinal cord damage occurs, as with the KED device, or when just concussive force is used, as with the Zephyr. The shorter latency to brain death with CD compared to the KED has previously also been found for older broilers, at 36, 42 and 43 days of age (Jacobs et al., 2019).

2.5.3 Success rate and reliability

The reliability of a device, in that it will always render the bird insensible and dead, is an important factor in the efficacy of a euthanasia method, and for the decision of which euthanasia method to implement for on-farm use. The misfiring of the Zephyr only occurred once in this experiment, with a second application necessary for euthanasia, however a higher incidence of misfires and unsuccessful kills were noted with the Zephyr in other experiments by this author (Chapter 3) and in previously published research (Erasmus et al., 2010a; Woolcott et al., 2018a). Similarly, other NPCD and penetrating captive bolt devices (Gregory and Wotton, 1990; Martin et al., 2016) have also been found to have unsuccessful euthanasia attempts. Published success

rates have ranged from 83% to 98% success when the Zephyr was used on turkeys (Erasmus et al., 2010a; Woolcott et al., 2018a), a 89% success rate for the TED when used on turkeys (Erasmus et al., 2010a), and 72% for a penetrating captive device (Martin et al., 2016). These unsuccessful attempts have been attributed to issues of device placement resulting in insufficient damage to the necessary brain regions (Erasmus et al., 2010a; Casey-Trott et al., 2013; 2014; Martin et al., 2016; Woolcott et al., 2018a), a mismatch between device capabilities (i.e. bolt force), bird species, sizes or age (Erasmus et al., 2010a; Woolcott et al., 2018a), and device malfunction, such as loss of air pressure reducing bolt force (Woolcott et al., 2018a) or device jamming.

The 100% efficacy rate seen with the CD suggests that this method is reliable for broilers throughout the production cycle, although the latency to insensibility is longer than with the Zephyr. Multiple other studies that also demonstrated CD, with proper training, to have a reliably high success, with CD having a 100% success rate for both broilers and layers (Martin et al., 2016) and 100% success rate seen in young turkeys in two separate studies investigating turkeys at 1 and 3 weeks of age (Erasmus et al., 2010a, Woolcott, 2017). The KED resulted in death 100% of the time, but the partial severing of the spinal cord extended the time to insensibility and death. Our findings in regards to the efficacy of the KED device differ from previous research investigating the KED as a euthanasia device for young turkeys. Woolcott and colleagues (2018a) found the KED to be unsuccessful and inappropriate for use for turkeys at 1 week of age, but also saw a high euthanasia success rate, similar to that found in our study, for turkeys at 3 weeks of age (Woolcott, 2017). The inability of the KED to successfully sever the spinal cord and kill turkeys at 1 week of age was attributed to the birds being too small for the device (Woolcott, 2017). However, the use of KED on broilers at 7 days in the current study had a euthanasia success rate of 100%. This suggests that the fact that the KED was unable to successfully euthanize young turkeys may be due to species specific issues resulting from a difference in body morphology, rather than just the small size of the birds.

When making decisions about the welfare of euthanasia methods, it is important to evaluate not only the time to unconsciousness and death, but also the success rate and the extent of excessive or additional tissue damage. The KED took the longest for insensibility and death to occur and may function by pushing apart of vertebrae rather than a dislocation by pulling the

vertebrae apart. Furthermore, it also had the highest number of severe fractures within the spinal column that are a possible cause for concern. Cervical dislocation results in a relatively rapid death with a 100% success rate, whilst the Zephyr resulted in a fast loss of consciousness, but occasionally misfires and requires a reshooting or application of a secondary method of euthanasia. Generally, all methods investigated in this study were reasonably effective and reliable at inducing onset of insensibility and death. Regardless of the method, it is important that it is performed by a trained person who is confident in performing the method, that death is verified and a secondary euthanasia method is used if required.

2.6 Conclusion

Overall, the welfare impact of a euthanasia method, as well as the decisions about which method is appropriate for on-farm usage, is dependent on a short latency to insensibility and death, the success rate of the methodology and the comfort and skill level of the person performing euthanasia with the particular euthanasia method. Although, none of the methods fully met all of the criteria above, all of the methods tested were reasonably effective for the euthanasia of broiler chickens. The Zephyr results in concussive damage to the skull and brain, resulting in tissue damage and hemorrhages that are sufficient to render the brainstem (and entire brain) non-functional. The concussive force of the Zephyr also resulted in a rapid insensibility, often within two seconds of the euthanasia application. The device occasionally misfires which requires either a secondary euthanasia attempt or a secondary euthanasia method. The twist and stretch action of CD was effective at severing of the spinal cord and carotid arteries, with a large separation distance, a dislocation close to the skull and high hemorrhaging scores signifying large volumes of blood loss, indicating cerebral ischemia and destruction of neural tissue. The histological evidence of hemorrhage in the brain also indicates a concussion of the brainstem. This combination of physical damage to the brainstem and spinal cord, concussion and cerebral ischemia resulted in the quickest time to death when CD was used, but time to loss of consciousness was longer than with Zephyr. Mechanical cervical dislocation with KED resulted in the longest latencies to insensibility and death. It also showed a reduced distance of separation, dislocations lower down the vertebral column, higher percentage of partial spinal cord transections and the higher number and severity of the fractures, and an absence of hemorrhages within the brain. This indicates that the KED may not be equivalent to CD, and may not always be efficacious at severing the spinal cord. However, it is a very simple method

to teach, and in some cases, may result in culling birds on a more timely manner than when other methods are required which the operator may not be as comfortable with or able to perform.

2.7 Acknowledgements

The author would like to thank the Saskatchewan producers that participated in the study, as well as, Chicken Farmers of Saskatchewan, Poultry Industry Council, Canadian Poultry Research Council, Egg Farmers of Canada, Hybrid Turkeys, the Ontario Ministry of Agriculture, Food and Rural Affairs, and Natural Sciences and Engineering Research Council for funding this study.

3.0 Chapter Three - Assessing the effect of dehydration on the euthanasia and the efficacy of on-farm euthanasia methods for broilers throughout the production cycle.

Birds requiring euthanasia are often dehydrated, and during previous research, it was noted that severely dehydrated birds responded to euthanasia differently. However, the majority of available research into on-farm euthanasia methods is based on science that used healthy or non-dehydrated broilers. To ensure the research from healthy birds is applicable to dehydrated birds, scientific knowledge on how dehydration affects euthanasia is needed. The objective of this chapter was to evaluate how dehydration impacts the euthanasia process and whether it affects the efficacy of on-farm euthanasia methods. Chapter 3 evaluated the effect of different levels of water deprivation on euthanasia by assessing behavioural and reflexive responses, as well as gross pathology, radiology and histology.

3.1 Abstract

Within a poultry flock, the birds that require euthanasia for reasons of illness are often dehydrated, particularly if reaching drinkers is not possible. The objective of this experiment was to isolate the effect of dehydration and investigate its effect on the efficacy of commercially available euthanasia methods. Broilers (n=179) at 8, 22, 36 and 50 days of age, were water deprived for 0, 24, 48 or 72 hours and then euthanized via manual cervical dislocation, mechanical cervical dislocation (Koechner Euthanasia Device), or a non-penetrative captive bolt (Zephyr EXL). Dehydration level was confirmed using skin turgor and packed cell volume. The efficacy of euthanasia was measured using the latency to loss of indicators of insensibility (pupillary light, palpebral blink and nictitating membrane reflex) and of death (feather erection (FE), cloacal winking (CW) and convulsions (CN)). In addition, macroscopic scoring of damage to the cranial-cervical region via radiography, gross pathology and histology was conducted. Main effects of euthanasia method and water deprivation level were analyzed by one-way analyses of variance (CRD, PROC MIXED, SAS 9.4) with a-priori contrasts to assess dehydrated versus non-dehydrated birds. Regression analyses (PROC REG, PROC RSREG) determined the effect of water deprivation time on behavioural indicator data. Water deprivation level had no effect on indicators of insensibility. Time to death as measured by FE, CW and CN was positively impacted by level of dehydration, either in a linear (FE; 22, 36 days, CW; 22 days, CN; 36 days) or quadratic (CW; 36, 50 days, CN; 22, 50 days) relationship. At 8 days of age, water deprivation level had an effect on subcutaneous hemorrhage in the neck and skin laceration, with scores decreasing as water deprivation increased. Water deprivation level only had an effect on the presence of hemorrhages within the brainstem. Overall, dehydration resulted in an increased latency to the onset of death, but did not affect the time to the onset of insensibility, with no interaction between euthanasia methods and dehydration status.

3.2 Introduction

The timely culling of diseased, injured or unfit birds during broiler production is essential in good farm management to ensure the welfare of birds. Animal care guidelines (NFACC, 2016a) for poultry require producers to take appropriate action to end the life and suffering of animals that are moribund and without prospect of recovery to minimize or eliminate pain or distress (Erasmus et al., 2010a,b,c; AVMA, 2013; Thornber et al., 2014; Cors et al., 2015; NFACC, 2016a). Within poultry flocks, the birds that require euthanasia are often found to be dehydrated, with dehydration being a concomitant factor to the birds' suffering. Dehydration is a welfare concern within a poultry flock as it results in suffering and distress and could exasperate the suffering from any underlying conditions.

Dehydrated birds can result from a number of issues, including disruptions in water supply. However, our focus in this paper centers on those birds that are destined for culling within the flock, as these birds may be unable to access, reach or utilize the drinkers and consume water (Butterworth et al., 2002; Manning et al., 2007; Sprenger et al., 2009; Rault et al., 2016). Within poultry production systems, birds are often provided water via nipple drinkers. These are raised bi-weekly as the birds grow to ensure that as the birds increase in size, they can still manipulate the nipples. The management practice of raising drinkers, whether nipple drinkers or bell drinkers, will result in water deprivation if the birds are unable to reach or operate the drinkers (Butterworth et al., 2002; Gregory, 2004; Savory, 2010). Undersized birds, or runts, are often dehydrated, as the raising of the drinkers means they are no longer able to reach the water source (Butterworth et al., 2002; Gregory, 2004; Savory, 2010). Disabilities or injuries that hinder mobility also often result in dehydration, as the birds are unable to maneuver sufficiently to access the waterers or are unable to reach and operate them. Research by Butterworth and colleagues (2002) showed that lameness impacted dehydration, with birds with severe gait abnormalities having higher plasma osmolality than those without. Lethargy or disability due to disease, social stress and overheating all also result in difficulty accessing the drinkers and thus water deprivation and dehydration (Gregory, 2004; Butterworth and Weeks, 2010; Savory, 2010). Dehydration is often either the cause for culling or found as concomitant to the reason for cull.

Water is often described as an essential component of the daily nutrient requirements of birds, as it is vital to life-sustaining processes and metabolic function (Leeson et al., 2007; Skomorucha et al., 2008; Castro et al., 2009; Viola et al., 2009; van der Klis and de Lange, 2013). Many aspects of metabolism are dependent on water, such as the control of body temperature, digestion, absorption and transport of nutrients from food, and the elimination of waste products (Manning et al., 2007). As water is essential to life, birds need to consume sufficient water to meet both the basal water need and to compensate for any additional water loss. Water deprivation and dehydration have been shown to result in a host of health issues that result in suffering and distress (Vanderhasselt et al., 2013), thus further negatively impacting bird welfare. The reduction in available body water reduces blood volume and circulation (Leeson et al., 2007; Viola et al, 2009), resulting in health issues such as increased body temperature and susceptibility to heat stress, metabolic acidosis, arrhythmias, circulatory failure, damage to the nervous system (Leeson et al., 2007) and toxemia (Bierer et al, 1965; Leeson et al., 2007). It has also been implicated in pathologies such as nephrosis and visceral gout, proventriculitis (Bierer et al., 1965) and cyanosis of the comb (Bierer et al., 1965; 1966). Dehydration also increases the likelihood of bacterial infections such as salmonella (Bierer et al., 1966). Water deprivation is a stressor and when combined with the stress of infection or disease, it can have an additive effect and intensify not only the eventual pathogenesis and clinical presentation of the infection or disease, but also the resulting suffering and the response to the stressor (Augustine, 1982). When over 10% of total body water is lost, dehydration is thought to be severe for the majority of animals, including poultry (Reece, 2015a), and water deprivation that results in a loss of 45% of body water will result in mortality (Leeson et al., 2007).

Euthanasia aims to result in a rapid loss of consciousness swiftly followed by death, with minimal pain or distress. The ability to induce a rapid insensibility and death can be measured by recording the latency to loss of involuntary behaviours and reflexive indicators of insensibility and death. These indicators have been validated and utilized in a wide range of previous research (Gerritzen et al., 2004; Erasmus et al., 2010a,b,c; Sandercock et al., 2014; Martin, 2015; Woolcott, 2017; Woolcott et al., 2018a). Cranial reflexes or brainstem reflexes, such as the corneal or palpebral blink reflex, the pupillary light reflex and nictitating membrane reflex, are indicators of insensibility and/or brain death (Erasmus et al., 2010b; Verhoeven et al., 2015; Terlouw et al., 2016b). These reflexes indicate whether there is an impairment to brainstem

functioning, as a disruption to the brain stem will inhibit cranial nerve functioning and result in the absence of brainstem reflexes. The cessation of rhythmic breathing, cloacal winking, convulsions and the occurrence of feather erection, are all indicators that death has occurred (Dawson et al., 2007; Dawson et al., 2009; Erasmus et al., 2010a,b; Martin et al., 2016; Terlouw et al., 2016b; Verhoeven et al., 2016). Rhythmic breathing is an involuntary behaviour under the autonomic regulation of a respiratory control centre in the brainstem (Martin et al., 2016; Terlouw et al., 2016b). An absence or dysregulation of rhythmic breathing means a disruption to the respiratory centres neurological control over respiration, which occurs when the brainstem is rendered non-functional and indicates brain death has occurred (Martin et al., 2016; Terlouw et al., 2016b). The action of cloacal winking is controlled by muscles that are innervated by nerves regulated by the brainstem, and the cessation of cloacal winking indicates the brainstem is no longer functional and that brain death has occurred (Erasmus et al., 2010b; Martin et al., 2016). The cessation of convulsions indicates the termination of brain activity and that brain death has occurred (Dawson et al., 2007; Dawson et al., 2009; Erasmus et al., 2010a,b; Martin, 2015; Martin et al., 2016; Verhoeven et al., 2016). Feather erection in an insensible bird is a sign of cardiac arrest or hypoperfusion (Heard, 2000; Erasmus et al., 2010b) and is an indicator that cardiovascular activity has ceased thus is an indicator of death (Casey-Trott et al., 2013, 2014).

The pathophysiology of dehydration and the concomitant effect of dehydration on infection, disease and injury, suggests that it could affect the euthanasia process. This possible effect of dehydration on euthanasia requires further study to understand the exact manner in which dehydration influences the occurrence of insensibility and death resultant from euthanasia. Furthermore, the majority of scientific evidence and knowledge available on poultry euthanasia originates from research performed on healthy or end-of-cycle birds and not those with visible clinical indications of disease (Gregory and Wotton, 1990; Raj and O'Callaghan, 2001; Cors et al., 2015; Martin et al., 2016). Thus to ensure research on euthanasia derived from healthy birds is applicable to birds with dehydration as a comorbidity, the objective of this research is to investigate the effect of dehydration on the euthanasia process and the efficacy of euthanasia methods throughout the production cycle of commercial broilers. It was hypothesized that dehydration will increase both the time to insensibility and the time to death, as water deprivation will result in physiological changes throughout the body that will affect the process via which consciousness is lost and death occurs.

3.3 Materials and methods

3.3.1 Ethical note

The research was approved by the University of Saskatchewan Animal Care committee and adhered to the recommendations of the Canadian Council of Animal Care (1993, 2009).

3.3.2 Experimental design

The experiment designed as a two-way factorial analysis, with dehydration level (0, 24, 48 or 72 hours without access to water prior to euthanasia) and euthanasia method (manual cervical dislocation (CD), mechanical cervical dislocation with the Koechner Euthanasia Device (KED), or a non-penetrating captive bolt with the Zephyr EXL (Zephyr) as treatment factors. Euthanasia with these techniques, and resulting measures, were conducted with birds at 8, 22, 36 and 50 days of age.

3.3.2.1 Dehydration level

Water was withdrawn from birds at the various ages for a number of hours, to create four dehydration treatments. These included 0 (control), 24, 48 or 72 hours of water deprivation. The dehydration control birds (0 hours) had ad libitum access to water throughout the experiment until euthanasia. For the other dehydration level treatments, the birds were deprived access to water for either 24, 48, or 72 hours prior to euthanasia. The water deprivation was conducted by randomly selecting fourteen birds from a larger group at 24, 48 and 72 hours prior to the euthanasia. The birds were removed from the home floor pens, weighed, wing banded for identification and then placed into the dehydration pens, in which they had access to ad lib feed but no access to water. During the water deprivation period, the birds were closely monitored for seizures, unresponsiveness and general listlessness to ensure no unnecessary suffering occurred.

3.3.2.2 Euthanasia methods

The Zephyr-EXL (Bock Industries, Inc. (BI), Philipsburg, PA, USA) is a pneumatic non-penetrative captive bolt device and was used with the associated CO_2 power system (Bock Industries, Inc. (BI), Philipsburg, PA, USA). The captive bolt was utilized with birds that were 22, 36 and 50 days of age only.

The Koechner Euthanasia Device (Koechner Mfg. Co., INC. Tipton, MO, USA) was used for mechanical cervical dislocation. As per the manufacturer's specification, the KED-S, was

used for birds of 8 and 22 days of age, whilst the KED-B was used for birds of 36 and 50 days of age. The device is designed with the goal of having the closure of the device around the neck to dislocate the cervical vertebrae.

Manual cervical dislocation was performed on days 22, 36 and 60, by restraining the bird with one hand grasping the bird's leg and the other placed on the neck at the base of the skull. Ventrodorsal rotational force was applied to stretch the neck, resulting in a separation of the skull and spine. For the 8 day old birds, the manual cervical dislocation was performed by placing the bird's mandible over a narrow edge and pressing firmly on the back of the neck using the thumb to separate the skull from the spinal column. The sharp edge used was from a pail with a 3.5mm edge. All euthanasia methods were performed by personnel trained and confident with the euthanasia method.

3.3.2.3 Birds and Housing

A total of 200 mixed sex Ross 308 broiler chicks were split into four equal groups and housed in four identical pens (3m x 3m), containing one bell drinker (diameter of 45.7cm) for water access and one aluminum tube feeder (circumference of 112 cm). Feed and water were provided ad libitum. Pens contained straw bedding of approximately 7.5 to 10 cm depth. Two additional pens were used for water deprivation pens, which were identical to the housing pens with the exception of an absence of drinkers. Birds placed in these pens had ad libitum access to feed, but no access to water. For the first ten days, both the home and dehydration pens contained a cardboard brooding ring (circumference of 8m), and supplemental feeders were provided in the brooding rings for the first 7 days. A 175-watt heat lamp also provided supplemental heat for the first 14 days. The broilers were fed a three stage commercial broiler feeding program using 0.5 kg starter/bird, 1.5 kg grower/bird, and the balance finisher. Room temperature was set at 32.1°C for the initial brooding period and then gradually reduced to 21°C by day 25. Lighting was provided by incandescent bulbs, with a photophase light intensity of 40 lux and a scotophase intensity of 0 lux, from day 0 till day 4. Photophase intensity was reduced to 20 lux from day 5 until trial end. Chicks were started on 23 hours of daylight for day 0 and 1, with the light period being reduced by an hour each day until a day length of 18 hours was reached at day 6; this was maintained until the end of the experiment. Birds were kept up to 50 days of age.

3.3.3 Data collection

3.3.3.1 Ante mortem data collection - Dehydration measures

Prior to euthanasia, each bird was weighed and dehydration status measured via skin turgor and packed cell volume. Skin turgor was measured using the pinch test, by pinching the skin on the left thigh of the bird for 10s, and recording time taken from skin release to return from tented shape to its previous state (Vanderhasselt et al., 2013). For packed cell volume, a small nick was made to the brachial vein of the bird. A sample of blood was collected in a micro-hematocrit tube (Kimble Chase, Vineland, NJ, USA). Samples were centrifuged for 3 minutes in a micro-capillary centrifuge MB (International Equipment Co., Boston, MA, USA) and packed cell volume over total volume of the blood sample was measured using the StatSpin tube reader (Iris Sample Processing, Westwood, MA, USA).

3.3.3.2 Ante mortem data collection – Indicators of insensibility and death

Visual assessment was used to measure the latency to insensibility and death in birds immediately post-euthanasia. Birds were assessed from moment of euthanasia until the time at which behavioural indicators were lost. Indicators used were latency to loss of eye reflexes (pupillary light, palpebral blink and nictitating membrane reflex) and the latency to onset and cessation of convulsions, cloacal winking and feather erection. Birds were monitored continuously for the presence or absence of these indicators until the total cessation of reflexes. Once an indicator was found absent, the bird was reassessed to confirm absence of indicator and monitored for its possible return. If an indicator was not measurable, it was marked as missing. Birds were considered dead once all indicators had ceased. The visual assessment was performed by the same trained individual throughout the experiment to ensure consistency. A description of the reflexes and how they were measured can be found in Table 3.1.

Table 3. 1. Ethogram of reflexes and involuntary behaviours measured ante mortem after application of euthanasia method.

Measure	Description
Pupillary light reflex	Constriction of pupil in response to light being shone into eye
Palpebral blink reflex	Closing of eyelids (blinking) in response to approach or touching of cornea
Nictitating membrane reflex	Closure of the nictitating membrane in response to approach or touching of cornea and medial canthus
Cloacal winking	Opening and closing of vent
Feather erection	Global piloerection
Convulsions	Uncontrolled involuntary muscle contractions
	Clonic – Wing-flapping
	Tonic – Muscle rigidity with final leg pedalling and wing-flapping

Adapted from Erasmus et al., 2010b; Martin et al., 2016; Terlouw et al., 2016b; Verhoeven et al., 2016.

3.3.3.3 Post-mortem data collection - Radiography

Four radiographs were taken of the cranial-cervical region of each bird using a Faxitron 43855D (Faxitron Bioptics, LLC, Tuscon, AZ, USA), with a CR 30-X digitizer (Agfa Healthcare NV, Mortsel, BE). The radiographs captured the right and left lateral, dorsal and ventral views. Radiographs were assessed using RadiAnt Dicom Viewer 3.4.1 (Medixant, Poznan, Poland) and evaluated for presence, location and distance of vertebrae separation, and number and severity of fractures within the vertebral column via visual assessment and scoring. Vertebrae separation was assessed by the presence of a dislocation between two vertebrae or between the first vertebrae (C1) and the skull. The distance of separation was assessed as a score by the proportion of the distance between the dislocated vertebrae to the length of the axis (C2). This was done to eliminate artificial differences in the measurement due to variation in bird size (Table 3.2).

Table 3. 2. Radiograph scoring scale for fracture severity and vertebra separation distance as a result of euthanasia method application.

Measure	Score	Severity descriptor
Fracture Severity		
	0	No fracture present
	1	Incomplete fracture
	2	Complete fracture
	3	Compound fracture
Separation distance		
	0	No separation of vertebrae
	1	Distance of separation is half the length of axis
	2	Distance of separation is more than half or equal to the length of axis
	3	Distance of separation is more than the length of axis.

3.3.3.4 Post mortem data collection – Gross pathology

Gross pathological evaluations were performed on the cranial-cervical regions of each bird, and visual assessment and macroscopic scoring was used for skin lacerations, hemorrhaging, fracturing of the skull, and complete severing of the spinal cord (Table 3.4). The macroscopic scoring scales have been previously used in poultry euthanasia research (Woolcott et al., 2018a; Baker, Chapter 2). Lacerations to the skin were evaluated at the site of euthanasia method application and assessed by the presence of laceration and external hemorrhage due to euthanasia method on a 2-point scale (Table 3.3). Following the scoring, skin was removed from the cranial-cervical region, and the tissues superior to the skull and neck were assessed for subcutaneous hemorrhaging. Hemorrhaging was scored on a 4-point scale and assessed the amount of blood lost from the vessels and pooling in the surrounding tissues. The calvarium was then assessed for extent of skull fracture, on a 3-point scale which assessed whether fractures were complete and penetrating. The calvarium, dura and brain were removed, and both the ventral and dorsal sides of the brain were assessed for subdural hemorrhaging. The vertebral column was then evaluated for the presence of fractures and to establish whether the spinal cord was completely severed.

Table 3. 3. Macroscopic scoring scale for skin rupture, hemorrhage and skull fracture severity due to euthanasia method application.

Measure	Score	Severity descriptor
Skin Laceration		
	0	No laceration or skin break present
	1	Laceration or skin break with no external hemorrhage
	2	Laceration or skin break with external hemorrhage
Hemorrhage		
	0	No hemorrhage present
	1	<25% of the area covered by hemorrhage
	2	25-50% of the area covered by hemorrhage
	3	50-75% of the area covered by hemorrhage
	4	>75% of the area covered by hemorrhage
Skull fracture		
	0	No fracture present
	1	Depression/ incomplete fracture
	2	Complete fracture with no imbedded fragments
	3	Complete penetrating fracture with imbedded fragments

3.3.3.5 Post mortem data collection – Histology

The brain and spinal cord of a subsection of the birds (n=33 which included one bird from each of the treatment combinations [water deprivation level * euthanasia method] per age), were fixed in 10% buffered formalin for a minimum of 7 days. A sagittal section was taken of the fixed brain and a section of 1cm or more of both ends of the spinal cord at severing site (Figure 3.1). Brain and spinal cord sections were paraffin embedded, sectioned and hematoxylin/eosin stained. Each section was evaluated by an observer who was blind to treatment for the presence of subdural and parenchymal hemorrhage in the cerebrum/pallium, cerebellum, brain stem and spinal cord using an Optika B-290TB Microscope (Optika. Bergamo, Italy) (Figure 3.2).

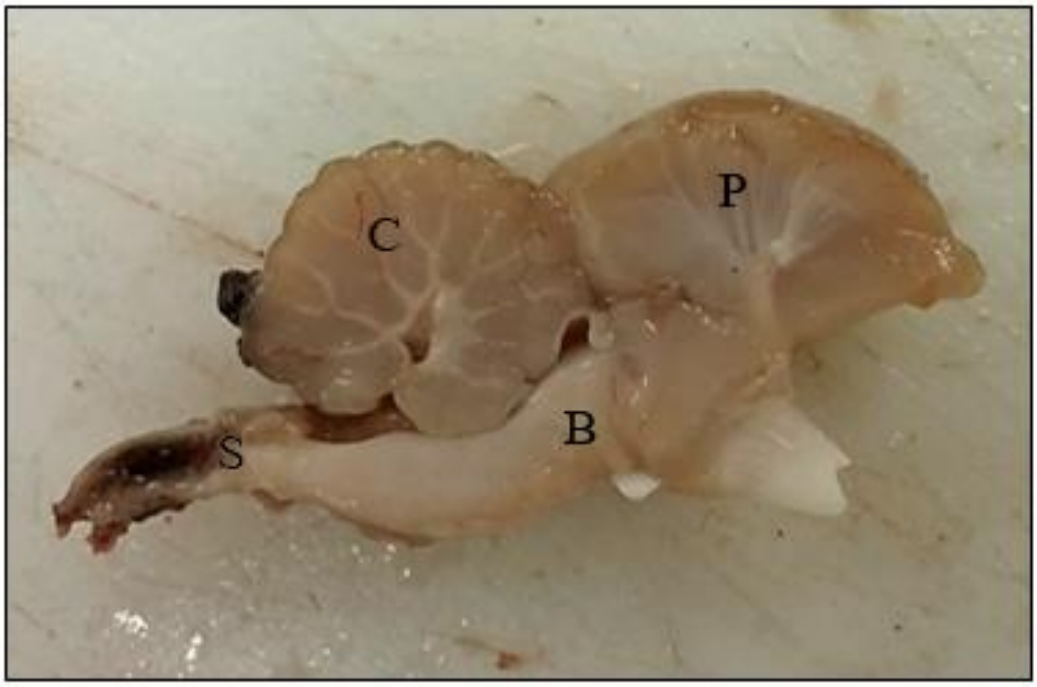

Figure 3. 1. A sagittal section of the avian brain and spinal cord , with the four sections of the brain evaluated for extradural and parenchymal hemorrhages. P: Pallium, C: Cerebellum, B: Brainstem, S: Spinal cord.

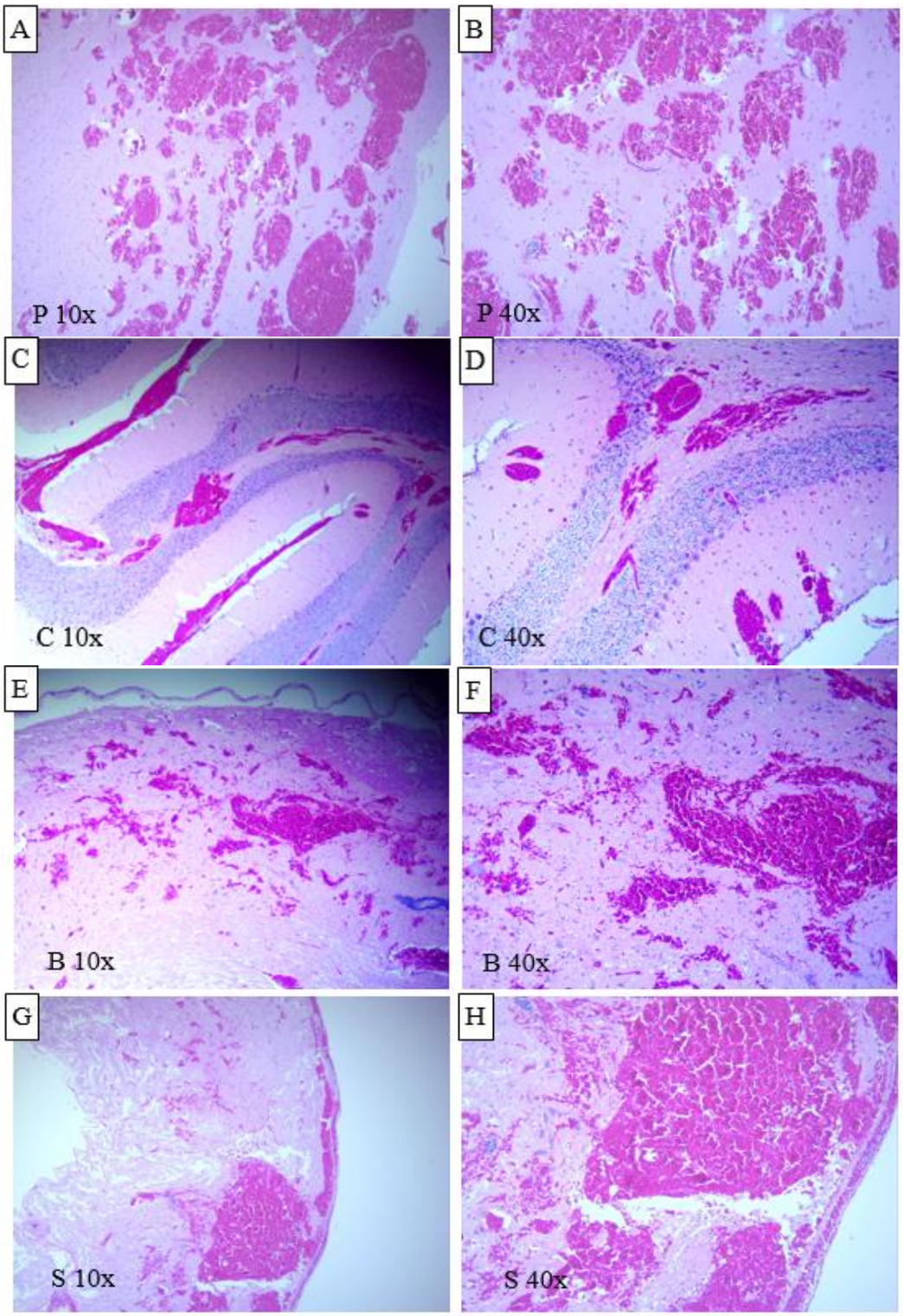

Figure 3. 2. Images of extradural and parenchymal hemorrhaging present in the pallium (A,B), cerebellum (C,D), brainstem (E,F) and spinal cord (G,H) of broilers euthanized with on-farm euthanasia methods, under 10x and 40x magnification. P: Pallium, C: Cerebellum, B: Brainstem, S: Spinal cord.

3.3.4 Statistical analyses

Prior to analyses, unsuccessful euthanasia attempts were removed from the data set to prevent skewing. Data for the dehydration status, the reflexes and behavioural indicators and histology were analyzed as a two-way factorial as a CRD for main effects of water deprivation level and euthanasia method using PROC MIXED, in SAS 9.4 (SAS Inst. Inc., Cary, NC, USA), with bird as experimental unit. A priori contrasts were performed to compare all water-deprived birds versus non-water-deprived for the reflex and behavioural data for each age separately. When differences were significant, means separation was performed with a Tukey-Kramer test.

Regression analyses were performed on the reflex and behaviour data to determine the effect of water deprivation level on the time to insensibility and death by age, with PROC REG and RSREG (SAS 9.4). Radiograph and macroscopic score data were non-parametric, thus rank transformed using the Friedman transformation in PROC RANK. Effects of water deprivation and euthanasia method were investigated via one-way analysis of variance (PROC MIXED) on the transformed data, with means separation via the Tukey-Kramer method. Differences were considered significant when the probability of difference was less than 0.05.

3.4 Results

3.4.1 Ante mortem data

3.4.1.1 Measures of dehydration

In the skin pinch test, at 8 days of age birds deprived of water for 72 hours took a longer time for skin to return to normal than birds that were not deprived of water (controls) and those water deprived for 24 hours or 48 hours. At 22 days, birds not deprived of water and those deprived for 24 hours had lower skin turgor then those with 72 hours of water deprivation. On day 50, non-water deprived birds differed from birds with 48 and 72 hours of water deprivation (Table 3.4). Packed cell volume at days 0, 22 and 36 were lower for birds that underwent 0 and 24 hours of water deprivation than those deprived for 72 hours. For all the ages, the extent of body weight loss was higher for birds deprived of water for 48 and 72 hours, than for bird deprived of water for 24 hours or those not deprived of water, and higher for birds deprived of water for 24 hours compared to those not water deprived. A quadratic relationship with water deprivation time was seen for both skin turgor, packed cell volume and body weight loss at all ages, with the highest values always noted in birds removed from water for 72 hours.

3.4.1.2 Indicators of insensibility and death

An interaction was found between euthanasia method and water deprivation level for pupillary light reflex at 8 days of age, and nictitating membrane reflex at 22 days (Table 3.5). At 8 days of age, the time to cessation of pupillary light reflex was longer for birds water deprived for 24 hours when euthanized by KED than for birds water deprived for 72 hours and euthanized by KED, and non-water deprived birds euthanized by CD. The latency to nictitating membrane reflex loss at 22 days was longest for non-water deprived birds euthanized with the KED, with the time differing from control birds and those deprived of water for 24 and 72 hours when euthanized by CD, and the Zephyr at all water deprivations levels. The shortest time to loss of nictitating membrane was noted with Zephyr regardless of water deprivation time. No effect of water deprivation level was seen for the time to loss of the three eye reflexes; pupillary light, palpebral blink and nictitating membrane reflex, at any of the ages (Table 3.5). A trend was noted at 36 days, when birds not deprived of water (0 hours) reached termination of pupillary light (P= 0.06) and nictitating membrane (P=0.09) reflexes in less time than any water-deprived birds. At 8 days, birds not deprived of water (0 hours) tended (P=0.09) to have a shorter latency to palpebral blink ending as compared to water- deprived birds. Time to loss of reflexes indicating insensibility differed with euthanasia method. With respect to pupillary light reflex, the longest latency was seen for birds euthanized by KED, and the shortest for those euthanized with the Zephyr, at 22, 36 and 50 days of age. With palpebral blink reflex and nictitating membrane reflex, the longest latencies were seen for birds euthanized by KED, and for both indicators at 22 days of age the shortest latencies were seen for birds euthanized by Zephyr.

The indicators of death, feather erection, cloacal winking or convulsions, did not demonstrate an interaction between euthanasia method and water deprivation level at any age (Table 3.6). The time to feather erection differed in broilers exposed to 72 hours of water deprivation compared to the non-water deprived (0 hour) birds at 36 days of age. The latency to onset of convulsions was shorter for birds not exposed to water deprivation than those deprived for 48 or 72 hours at 50 days of age. A difference was found for latency to cessation of cloacal winking between non-water deprived birds (0 hours) and birds deprived for 24 and 72 hours at

Table 3. 4. Effect of water deprivation level on skin turgor as measured by the skin pinch test and packed cell volume in broiler chickens at 8, 22, 36 and 50 days of age.

Age (d)	n	Water Deprivation Level (h)				*P* value	SEM[1]	Regression	
		0	24	48	72			*P* value	Equation
Skin turgor (s)									
8	35	1.7^{b}	4.7^{b}	11.9^{b}	22.5^{a}	<0.01	0.88	$<0.01^{Q}$	$y=0.0018x^2+0.129x+1.13$
22	48	1.4^{b}	2.3^{b}	4.0^{ab}	5.2^{a}	<0.01	0.62	$<0.01^{Q}$	$y=0.0006x^2+0.008x+1.56$
36	45	1.0	1.6	2.2	2.8	0.06	0.39	0.02^{Q}	$y=-0.0001x^2+0.04x+0.98$
50	45	1.4^{c}	2.0^{bc}	2.6^{a}	2.4^{ab}	<0.01	0.14	$<0.01^{Q}$	$y=-0.0004x^2+0.05x+1.29$
Packed cell volume (%)									
8	35	37.4^{b}	38.7^{b}	47.0^{a}	49.7^{a}	<0.01	2.06	$<0.01^{Q}$	$y=0.0007x^2+0.151x+36.38$
22	47	30.4^{d}	35.7^{c}	39.7^{b}	43.2^{a}	<0.01	1.01	$<0.01^{Q}$	$y=-0.0004x^2+0.201x+30.72$
36	45	35.7^{bc}	36.2^{c}	40.4^{ab}	43.4^{a}	<0.01	1.07	$<0.01^{Q}$	$y=0.0013x^2+0.029x+35.47$
50	44	32.4^{b}	36.5^{ab}	42.0^{a}	39.7^{ab}	<0.01	1.53	$<0.01^{Q}$	$y=-0.0025x^2+0.306x+31.36$
Body weight loss (g)									
8	35	0^{c}	21.7^{b}	31.4^{a}	32.0^{a}	<0.01	1.04	$<0.01^{Q}$	$y=-0.0091x^2+1.094x+0.28$
22	48	0^{c}	104.8^{b}	146.6^{a}	165.5_{a}	<0.01	5.20	$<0.01^{Q}$	$y=-0.0361x^2+4.808x+4.48$
36	45	0^{c}	236.6^{b}	341.8^{a}	379.5^{a}	<0.01	13.87	$<0.01^{Q}$	$y=-0.0854x^2+11.288x+6.49$
50	45	0^{c}	336.4^{b}	537.1^{a}	623.2^{a}	<0.01	24.70	$<0.01^{Q}$	$y=-0.1078x^2+16.368x+2.63$

[a-d] Means with common letter within a row and main effect do not differ significantly (P≤0.05).

[1]Standard error of the mean.

*Non = non-water-deprived, WD= Water-deprived.

[Q] = Quadratic.

36 days of age, and between non-water deprived birds (0 hours) and those deprived for 24 and 48 hours at 50 days of age. The latency to feather erection was longest for birds euthanized by KED compared to CD and Zephyr at 36 and 50 days. Similarly, the latency to cloacal winking was longer for birds euthanized by KED than those euthanized by CD and Zephyr at 50 days, while at 36 days, the latency to cloacal winking was longer for bird euthanized with the KED than by CD. The time to convulsions was longer for birds euthanized by KED than by CD at both 36 and 50 days of age.

When all the water deprivation levels were contrasted to the non-water deprived controls, birds showed latency to performance of feather erection at 36 and 50 days, convulsions at 22, 36 and 50 days, and cloacal winking at 22, 36 and 50 days with the latencies being shorter for the dehydrated birds (Table 3.7). At 22 and 36 days of age there was a positive linear increase in latency to feather erection with increasing water deprivation levels. The latency to termination of cloacal winking occurred in a quadratic relationship to water deprivation at 50 days of age (longest latency with 48 hours deprivation), a trend (P=0.05) towards a quadratic relationship at 36 days (longest latency with 72 hours) deprivation and a positive linear relationship at 22 days. For convulsions, water deprivation resulted in a quadratic relationship with time to cessation of convulsions at 22 and 50 days, with the longest latency with 72 hours deprivation, and a trend (P=0.10) towards a positive linear relationship at 36 days.

Table 3. 5. Effect of water deprivation level on time from euthanasia performance to cessation of pupillary light -, palpebral blink - and nictitating membrane reflex in broiler chickens at 8, 22, 36 and 50 days of age.

Age		Euthanasia method (E)[1]				Water deprivation level (h) (W)					E*W	
(d)	n	CD	KED	Zephyr	*P* value	0	24	48	72	*P* value	*P* value	SEM[2]
Pupillary light reflex (s)												
8	29	63.4	72.5	-	0.11	58.4	83.2	65.6	61.5	0.06	0.03	8.24
22	42	57.9^{b}	79.1^{a}	1.0^{c}	<0.01	46.5	47.8	45.8	44.1	0.95	0.69	12.47
36	38	57.7^{b}	91.5^{a}	12.4^{c}	<0.01	37.7	56.2	66.0	57.4	0.06	0.78	11.47
50	39	65.4^{b}	105.9^{a}	12.1^{c}	<0.01	55.6	67.6	64.4	67.7	0.84	0.92	14.83
Palpebral blink reflex (s)												
8	29	15.6^{b}	22.8^{a}	-	0.20	12.2	23.5	21.1	17.9	0.09	0.53	3.27
22	41	23.7^{b}	33.1^{a}	1.0^{c}	<0.01	18.8	19.1	19.1	19.4	0.99	0.88	5.55
36	39	14.0^{b}	28.4^{a}	12.9^{b}	<0.01	12.0	19.9	20.3	20.1	0.14	0.56	3.55
50	39	13.9^{b}	27.7^{a}	12.1^{b}	0.01	12.4	18.6	17.3	21.8	0.68	0.42	3.91
Nictitating membrane reflex (s)												
8	29	48.1	61.1	-	0.21	64.2	61.0	51.9	45.9	0.82	0.38	16.38
22	40	36.9^{b}	55.9^{a}	1.0^{c}	<0.01	30.3	32.3	32.6	28.6	0.43	0.04	9.43
36	39	21.4^{b}	77.4^{a}	14.8^{b}	<0.01	21.5	38.8	45.0	45.4	0.09	0.58	10.44
50	39	27.4^{b}	93.5^{a}	17.7^{b}	<0.01	34.0	48.1	51.5	51.9	0.71	0.80	14.45

$^{a-c}$ Means with common letter within a row and main effect do not differ significantly (P≤0.05).

[1]KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt

[2]Standard error of the mean.

Table 3. 6. Effect of water deprivation level on the latency to cessation of feather erection, convulsions and cloacal winking in broiler chickens at 8, 22, 36 and 50 days of age.

Age (d)	n	Euthanasia method (E)			*P* value	Water deprivation level (h) (W)				*P* value	E*W	
		CD	KED	ZEP		0	24	48	72		*P* value	SEM[1]
Feather erection (s)												
8	29	-	-	-	-	-	-	-	-	-	-	-
22	38	69.1	66.5	53.4	0.11	46.6	63.2	60.2	71.9	0.27	0.87	6.38
36	39	52.5^{b}	77.9^{a}	43.5^{b}	<0.01	41.3^{b}	59.5^{ab}	61.4^{ab}	66.3^{a}	0.02	0.14	6.42
50	39	58.1^{b}	82.8^{a}	57.1^{b}	0.01	48.0	73.8	64.4	70.5	0.20	0.80	6.94
Convulsions (s)												
8	29	109.4	113.3	-	0.37	125.8	110.0	108.9	106.5	0.80	0.17	21.96
22	42	108.6	114.6	101.7	0.22	85.5	105.3	113.2	117.8	0.11	0.36	8.91
36	39	75.6^{b}	105.9^{a}	98.4^{ab}	<0.01	73.2	94.8	96.8	98.1	<0.01	0.35	6.98
50	39	91.5^{b}	120.9^{a}	92.7^{ab}	<0.01	66.0^{b}	100.9^{ab}	108.9^{a}	113.4^{a}	0.04	0.85	9.08
Cloacal winking (s)												
8	24	108.7	130.2	-	0.66	126.2	120.2	121.9	99.3	0.97	0.76	16.68
22	41	118.7	123.3	112.9	0.17	99.7	116.7	122.1	126.6	0.09	0.22	8.04
36	39	96.0^{b}	124.1^{a}	115.0^{ab}	<0.01	88.5^{b}	116.8^{a}	112.5^{ab}	117.2^{a}	0.06	0.12	6.21
50	38	107.6^{b}	140.0^{a}	110.4^{b}	0.02	86.2^{b}	123.4^{a}	127.0^{a}	122.8^{ab}	0.02	0.88	7.23

^{a,b} Means with common letter within a row and main effect do not differ significantly (P≤0.05).

KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt

*Non = non-water-deprived, WD = water-deprived

[1]Standard error of the mean.

^{L} = Linear, ^{Q} = Quadratic

Table 3. 7. Effect of water deprivation level on the latency to cessation of feather erection, convulsions and cloacal winking in broiler chickens at 8, 22, 36 and 50 days of age.

Age		Contrast			Regression	
(d)	n	Non-water deprived	Water deprived	*P* value	*P* value	Equation
Feather erection (s)						
8	29	-	-	-	-	-
22	38	46.6	65.1	0.17	0.03^{L}	*y=0.28x+50.99*
36	39	41.3^{b}	62.4^{a}	<0.01	0.04^{L}	*y=0.28x+47.88*
50	39	45.5^{b}	69.6^{a}	0.05	NS	-
Convulsions (s)						
8	29	125.8	108.5	0.64	NS	-
22	42	85.5^{b}	112.1^{a}	0.02	0.04^{Q}	$y=-0.006x^2+0.89x+86.32$
36	39	73.2^{b}	96.6^{a}	<0.01	0.09^{L}	*y=0.264x+82.34*
50	39	65.5^{b}	107.7^{a}	<0.01	0.01^{Q}	$y=-0.013x^2+1.51x+68.51$
Cloacal winking (s)						
8	24	126.2	113.8	0.61	NS	-
22	41	99.7^{b}	121.8^{a}	0.03	0.02^{L}	*y= 0.33x+105.22*
36	39	88.5^{b}	115.5^{a}	<0.01	0.05^{Q}	$y=-0.010x^2+1.04x+92.14$
50	38	84.7^{b}	124.4^{a}	<0.01	0.03^{Q}	$y=-0.017x^2+1.69x+88.98$

^{a,b} Means with common letter within a row do not differ significantly (P≤0.05).
KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt
[1]Standard error of the mean.
^{L} = Linear, ^{Q} = Quadratic

3.4.2 Post mortem data

3.4.2.1 Radiography

The radiography data did not result in an interaction between water deprivation level and euthanasia method, although a trend (P=0.07) for an interaction was noted for the separation distance score of birds at 50 days of age (Table 3.8). Radiography data also showed no effect of water deprivation level on scores for vertebrae fracture severity within the spinal column or separation distance at the site of dislocation. Euthanasia method did result in an effect on separation distance, with distance of separation being largest for CD, followed by KED and then Zephyr (which is not expected to result in vertebral separation), at 22, 36 and 50 days. The fracture severity was the highest for broilers euthanized with the KED, and was different from both broilers euthanized with either the Zephyr and CD at 8, 22, and 50 days, and from Zephyr at 36 days.

3.4.2.2 Gross pathology

Water deprivation and euthanasia method showed no interaction effect on any of the gross pathology measures, although scores for subcutaneous hemorrhages on the neck of birds at 8 days of age demonstrated a trend (P=0.08) for an interaction (Table 3.9 and Table 3.10). Water deprivation affected the scores for subcutaneous hemorrhage on the neck and skin lacerations at 8 days of age, with scores for broilers not water-withdrawn being higher than the scores for broilers water deprived for 48 or 72 hours. There was no effect of water deprivation of the gross anatomy scores for subdural hemorrhaging, subcutaneous hemorrhaging on the head, and skull fracture at all ages, or for subcutaneous hemorrhaging on the neck and skin laceration at 22, 35 and 50 days of age. Euthanasia method resulted in a difference in subdural hemorrhage, subcutaneous hemorrhage on the head and skull fracture scores, with scores being highest for birds euthanized with the Zephyr compared to both CD and KED, at 22, 35 and 50 days. Differences were also found for skin laceration score among euthanasia methods at 22, 35 and 50 days, with the higher scores found for birds euthanized with Zephyr and KED than for those euthanized by CD. Subcutaneous hemorrhage of the neck scores differed with euthanasia methods, at 8 and 50 days the highest scores seen for CD, whilst at 22 and 35 days the highest scores were seen for both CD and KED.

Table 3. 8. Effect of water deprivation and euthanasia method on fracture severity in spinal column and separation distance scores resulting from euthanasia in broiler chickens at 8, 22, 36 and 50 days of age.

Age		Euthanasia method (E)*					Water deprivation level (h) (W)						E*W
(d)	n	KED	CD	Zephyr	Control	*P* value	0	24	48	72	*P* value	SEM[1]	*P* value
Fracture severity[2]													
8	35	1.2[a]	0.5[ab]	-	0.0[b]	0.01	0.8	0.9	0.8	0.4	0.76	0.18	0.18
22	42	2.1[a]	0.3[b]	0.0[b]	0.0[b]	<0.01	-	1.0	0.4	0.6	0.14	0.17	0.37
36	45	0.9[a]	0.6[ab]	0.0[b]	0.0[b]	0.03	0.3	0.4	0.2	0.8	0.60	0.13	0.92
50	45	1.2[a]	0.1[b]	0.0[b]	0.0[b]	0.01	0.4	0.4	0.5	0.4	0.97	0.14	0.99
Separation distance[3]													
8	35	1.0[b]	3.0[a]	-	0.0[c]	-	1.8	1.6	1.6	1.6	-	0.20	-
22	42	1.2[b]	3.0[a]	0.0[c]	0.0[c]	<0.01	-	1.2	1.2	1.1	0.72	0.20	0.87
36	45	0.9[b]	2.9[a]	0.0[c]	0.0[c]	<0.01	1.3	1.1	1.2	1.1	0.80	0.19	0.70
50	45	1.8[b]	3.0[a]	0.0[c]	0.0[c]	<0.01	2.4	1.5	1.2	1.4	0.11	0.20	0.07

[a-c] Means with common letter within a row and main effect do not differ significantly (P≤0.05).

*KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt, Control = T-61 injection.

[1]Standard error of the mean.

[2]Scores for fracture severity on 3-point scale: 0 = no fracture, 1 = incomplete fracture, 2 = complete fracture, 3 = compound fracture.

[3] Scores for separation distance on 3-point scale: 0 = no separation 1 = ≤ ½ axis length, 2 = ≥ ½ axis length or = axis length, 3 = ≥ axis length.

Table 3. 9. Effect of water deprivation and euthanasia method on subdural hemorrhaging, subcutaneous hemorrhaging on the head and neck as a result of euthanasia in broiler chickens at 8, 22, 36 and 50 days of age.

Age		Euthanasia method (E)*					Water deprivation level (h) (W)						E*W
(d)	n	KED	CD	Zephyr	Control	*P* value	0	24	48	72	*P* value	SEM_1	*P* value
Subdural hemorrhaging[2]													
8	35	0.0	0.0	-	0.0	-	0.0	0.0	0.0	0.0	-	0.00	-
22	48	0.1[b]	0.2[b]	3.4[a]	0.0[b]	<0.01	0.8	1.1	0.9	1.2	0.18	0.22	0.72
36	45	0.1[b]	0.2[b]	3.0[a]	0.0[b]	<0.01	1.2	0.8	0.7	0.7	0.62	0.20	0.52
50	44	0.3[b]	0.2[b]	2.9[a]	0.0[b]	<0.01	0.6	0.7	1.0	1.1	0.69	0.20	0.57
Subcutaneous hemorrhaging on dorsal head[2]													
8	35	0.0	0.0	-	0.0	-	0.0	0.0	0.0	0.0	-	0.00	-
22	48	0.1[b]	0.0[b]	3.9[a]	0.0[b]	<0.01	1.2	1.1	1.2	1.1	0.37	0.26	0.63
36	45	0.3[b]	0.1[b]	2.8[a]	0.2[b]	<0.01	1.0	1.1	0.6	0.8	0.43	0.19	0.76
50	45	1.5[b]	0.1[c]	3.3[a]	0.0[c]	<0.01	1.4	1.3	1.6	0.9	0.88	0.26	0.90
Subcutaneous hemorrhaging on neck[2]													
8	35	2.1[b]	3.6[a]	-	0.0[c]	<0.01	4.0[a]	2.7[ab]	2.0[b]	1.5[b]	<0.01	0.27	0.08
22	48	3.6[a]	3.7[a]	0.0[b]	0.0[b]	<0.01	2.5	2.3	2.0	2.0	0.57	0.28	0.81
36	45	3.6[a]	3.8[a]	0.0[b]	0.0[b]	<0.01	2.7	2.3	2.3	2.2	0.73	0.29	0.90
50	45	3.4[b]	4.0[a]	0.3[b]	0.0[b]	<0.01	3.2	2.5	2.0	2.4	0.90	0.29	0.87

[a-c] Means with common letter within a row and main effect do not differ significantly (P≤0.05).

[1]Standard error of the mean.

[2]Scores of proportion of tissue with hemorrhage: 0 = 0%, 1 = <25%, 2 = 25-50%, 3 = 50-75%, 4 = >75%.

Table 3. 10. Effect of water deprivation and euthanasia method on skull fracture and skin laceration as a result of euthanasia in broiler chickens at 8, 22, 36 and 50 days of age.

Age		Euthanasia method (E)*					Water deprivation level (h) (W)						E*W
(d)	n	KED	CD	Zephyr	Control	*P* value	0	24	48	72	*P* value	SEM_1	*P* value
Skull fracture[2]													
8	35	0.0	0.0	-	0.0	-	0.0	0.0	0.0	0.0	-	0.00	-
22	48	0.0^{b}	0.0^{b}	2.8^{a}	0.0^{b}	<0.01	0.8	0.8	0.8	0.8	0.34	0.19	0.42
36	45	0.0^{b}	0.0^{b}	2.0^{a}	0.0^{b}	<0.01	0.7	0.6	0.2	0.5	0.37	0.14	0.41
50	45	0.4^{b}	0.0^{b}	1.8^{a}	0.0^{b}	<0.01	0.0	0.5	0.7	0.8	0.14	0.16	0.49
Skin laceration[3]													
8	35	0.2	0.0	-	0.0	0.06	0.4^{a}	0.1^{ab}	0.0^{b}	0.0^{b}	0.04	0.05	0.23
22	48	1.6^{a}	0.3^{b}	1.0^{a}	0.0^{b}	<0.01	1.3	1.0	0.6	0.7	0.42	0.14	0.38
36	45	1.4^{a}	0.0^{b}	1.3^{a}	0.0^{b}	<0.01	1.3	0.7	0.5	0.8	0.40	0.15	0.44
50	45	1.6^{a}	0.0^{b}	1.6^{a}	0.0^{b}	<0.01	0.8	0.6	1.1	0.9	0.41	0.15	0.47

[a-c] Means with common letter within a row and main effect do not differ significantly (P≤0.05).

[1]Standard error of the mean.

[2]Scores for skull fracture: 0 = no fracture, 1 = depression/incomplete fracture, 2 = complete fracture with no imbedded fragments, 3 = complete penetrating fracture with imbedded fragments.

[3]Scores for skin laceration: 0 = no laceration, 1 = laceration with no external hemorrhage, 2 = laceration with external hemorrhage.

3.4.2.3 Histology

No interaction was noted between water deprivation and euthanasia method for hemorrhaging in any region of the brain (Table 3.11). Water deprivation had no impact on presence of hemorrhages in cerebrum, cerebellum and spinal cord. The brainstem showed that the percentage of broilers with extradural hemorrhages was higher for broilers withdrawn of water for 72 hours as compared to those withdrawn for 48 hours. No effect of water deprivation was found on total presence of hemorrhaging on the brain. Euthanasia methods resulted in a difference in presence of hemorrhages in the cerebrum and cerebellum. The presence of hemorrhaging in the cerebrum was also higher for the bird euthanized with Zephyr than those euthanized with the CD or KED, whereas the presence of hemorrhages in the cerebellum was higher for broiler euthanized with the Zephyr, followed by those euthanized by CD and finally by KED. No effect of euthanasia method on the presence of hemorrhage was seen for the brainstem and spinal cord. The data indicated that the total presence of hemorrhage was higher with use of the Zephyr versus the CD and KED.

3.4.3 Success rate

The overall success rate for both CD and KED was 100%, while the Zephyr had a success rate at first application of 88%. Of the 42 birds that were euthanized with the Zephyr, three required a second bolt to result in death successfully. Two additional euthanasia attempts with the Zephyr were unsuccessful, and a secondary method of euthanasia, CD, was employed. No effect of dehydration level on euthanasia success was seen, but the attempts requiring a second application attempt or that were successful occurred with one control bird, one bird water-deprived for 24 hours, two birds deprived for 48 hours, and one deprived for 72 hours.

Table 3. 11. Effect of water deprivation and euthanasia method on the percentage of broilers with extradural or parenchymal hemorrhaging present within the different regions of the brain.

% with		Euthanasia Method (E)*				Water Deprivation (h) (W)					E*W
hemorrhage	n	Zephyr	CD	KED	*P* value	24	48	72	*P* value	SEM[1]	*P* value
Cerebrum	33	88.9[a]	8.3[b]	0.0[b]	<0.01	27.7	36.6	18.2	0.20	7.87	0.49
Cerebellum	33	100.0[a]	41.7[b]	8.3[c]	<0.01	27.3	54.5	54.5	0.14	8.80	0.14
Brainstem	33	66.7	50.0	33.3	0.32	54.5[ab]	18.8[b]	72.7[a]	0.04	8.83	0.96
Spinal Cord	32	100.0	83.3	83.3	0.54	90.9	90.0	81.8	0.83	5.94	0.72
Total	33	86.1[a]	45.8[b]	31.2[b]	<0.01	50.0	47.7	56.8	0.58	4.91	0.76

[a-c] Means with common letter within a row and main effect do not differ significantly (P≤0.05).

*KED = mechanical cervical dislocation, CD = manual cervical dislocation, Zephyr = non-penetrative captive bolt.

[1]Standard error of the mean.

3.5 Discussion

Within a poultry flock, the moribund, ill and unfit birds that require euthanizing are often found to be deprived of water, and thus suffering from dehydration alongside the primary health concern. By inducing dehydration via water deprivation, it was possible to isolate the effects of dehydration and understand its impact on the success and the process of euthanasia via three different methods of commercial on-farm euthanasia methods. The effects of the euthanasia methods on the indicators of insensibility and death, as well as on the macroscopic, radiography and histology scores, replicated those seen in a previous experiment by these authors investigating these exact euthanasia methods (Baker, Thesis Chapter 2). With the shortest time to insensibility seen with the Zephyr, and the longest time to insensibility and death seen with the KED. Similar to the previous research, the post-mortem data demonstrated that the euthanasia methods resulted in death via concussive force, for the Zephyr, and severing of the spinal cord and carotid arteries, for the CD and KED. That the euthanasia methods show the same results as before indicate that dehydration did not affect the efficacy of one method more than the others.

Water deprivation did not affect the time to insensibility, but it did increase time to death. This could be due to how the body prioritises and compensates when dehydration occurs, and the organ system involved with maintaining consciousness and maintaining life. Consciousness is maintained by various regions of the brain (Reiner et al., 2005; Butler and Cotterill, 2006; Erasmus et al., 2010b), and insensibility occurs when there is a dysfunction in these areas of the brain (Terlouw et al., 2016a). Furthermore, the three eye reflexes used in this experiment to indicate insensibility involve cranial nerves and thus are direct indicators of the brain function (Erasmus et al., 2010b; Terlouw et al., 2010b). During dehydration, the body prioritises components that are essential to maintaining life, such as the brain, and will redirect electrolytes and fluids to these organs in an attempt to sustain the normal fluid volume (Gullans and Verbalis, 1993; Kempton et al., 2009), in a manner similar to the body prioritising oxygen to the brain in cases of hypoxia. If fluid is prioritised by the brain, it may result in the brain showing fewer effects of dehydration; thus the effects of water deprivation were less severe and less visible in this region. It may also mean that as the water deprivation is less apparent in the brain, the reflexive indicators of insensibility and time to insensibility in response to euthanasia are not affected by water deprivation. Death, however, involves the cessation of all life-sustaining functions and is dependent on respiratory and cardiac arrest, as well as the cessation of brain

function (Adams and Sheridan, 2008; Thornber et al., 2014; Cors et al., 2015). As this process involves multiple organ systems, all of which will be affected by dehydration in their own way, the impact of dehydration on death is larger than on insensibility. For example, as the blood volume decreases in response to dehydration this will reduce circulation (Leeson et al., 2007; Viola et al, 2009), lengthening the time needed for blood to travel through the body and to the necessary organs, thus increasing the time to death as the efficiency of the different organ systems and cells within the body is reduced. The effect of dehydration being most significant outside of the cranial region is mirrored in the gross pathology and histology scores. The lack of difference as impacted by water deprivation level in the macroscopic scores for damage to the head and histology scores for the cerebrum and cerebellum suggest that dehydration is preserving the brain and head, thus minimising the effect of dehydration on the latency to insensibility with euthanasia.

The brainstem was the only region of the brain that showed a response to water deprivation via the presence of hemorrhages, although the response did not seem to follow any clear pattern. However, this relationship could be explained by the importance of the brainstem and the manner in which the body tries to restore brain volume in response to dehydration over the different lengths of time (Gullans and Verbalis, 1993). The brainstem is a vital component in sustaining both consciousness and life within the avian brain (Erasmus et al., 2010b; Terlouw et al., 2016a). It is hypothesised that at 48 hours of deprivation, the reduction in plasma volume to compensate for water loss could mean there is less blood available for hemorrhaging than at 24 hours. As 48 hours of water deprivation would be a more acute dehydration, during which hemoconcentration occurs and there is a loss of total volume of fluid in the brain (without a loss of intracellular fluid) (Gullans and Verbalis, 1993). As dehydration continues and becomes more chronic, the body prioritizes increasing the volume of fluid within the cranial region (Gullans and Verbalis, 1993). Furthermore as the dehydration increases the integrity of the cells within the brain and the capillaries will have deteriorated, as intracellular fluid is lost, making them more vulnerable and easier to damage, resulting in increased hemorrhages represented in the higher hemorrhage scores at 72 hours.

The efficacy of a euthanasia method is not only dependent on its ability to induce a rapid loss of consciousness and death but also that it consistently results in a successful death without a

need for a repeat application of a euthanasia method (Baker, Thesis Chapter 2). During this study, the rate of euthanasia success was shown to be affected by the euthanasia method with the Zephyr being the only method requiring either a second application or the use of a secondary euthanasia method. The occurrence of unsuccessful euthanasia attempts was not high enough to determine whether there was an effect of water deprivation level.

3.6 Conclusion

Dehydration caused by water deprivation had no visible effect on the latency to insensibility, but did increase the latency to death at 22, 36 and 50 days of age. Dehydration did not affect the efficacy of the euthanasia methods, reflected both in the success rates and the scores for radiography, gross pathology and histology. As dehydration did not increase the time to insensibility and thus does not affect the time in which the birds could be conscious to possible distress associated with the euthanasia process or increase the negative damage that may result in pain or distress, it can be concluded that dehydration does not negatively impact the welfare of euthanasia. The difference seen for the time to death with the water deprivation, may be attributed to the body prioritising the distribution of water to the different compartments and organs throughout the body. Overall, the efficacy of the individual euthanasia methods was not affected by water deprivation.

3.7 Acknowledgements

The author would like to thank Chicken Farmers of Saskatchewan, Poultry Industry Council, Canadian Poultry Research Council, Egg Farmers of Canada, Hybrid Turkeys, the Ontario Ministry of Agriculture, Food and Rural Affairs, and Natural Sciences and Engineering Research Council for funding this study.

4.0 Chapter Four - Evaluation of carbon dioxide induction methods for the euthanasia of day-old cull broiler chicks

Chapter 4 focuses on the euthanasia of cull chicks on day of hatch. At present, there is little information available on the use of CO_2 for the euthanasia of neonates in hatcheries. Although there are some recommendations available for using CO_2 with adult birds, these may not be applicable to chicks in a hatchery setting as neonates have a higher CO_2 tolerance compared to adult birds. As no research has been published on the use of CO_2 for the euthanasia of neonate broilers, the objective of this chapter was to gain a scientific understanding of the use of CO_2 for neonates and to establish the welfare impact and efficacy of CO_2 gaseous euthanasia for culling broiler chicks. The research in Chapter 4 aimed to ascertain basic information regarding displacement rates, gas concentrations and exposure times appropriate for euthanizing neonates, and aimed to evaluate five induction methods on their efficacy and welfare impact by measuring distress and time to insensibility and death.

This chapter has been reformatted for inclusion in this thesis. The manuscript has been published in Poultry Science, and received Editor's Choice.

Baker, B.I., S. Torrey, T.M. Widowski, P.V. Turner, T.D. Knezacek, J. Nicholds, T.G. Crowe, and K. Schwean-Lardner. 2019. Evaluation of carbon dioxide induction methods for the euthanasia of day-old cull broiler chicks. Poult. Sci. 98:2043-2053. https://doi.org/10.3382/ps/pey581

4.1 Abstract

This study was conducted to evaluate the efficacy of five different CO_2 euthanasia induction techniques for day-old cull chicks in minimizing distress and inducing a rapid loss of sensibility and death. Each induction treatment was characterized for concentration change over time, maximum concentration, and time to reach maximum. Sixteen chicks were euthanized with the gradual treatments to establish validity of treatment. Then, all five treatments were evaluated for effect on distress, insensibility and death. Day-of-hatch cull chicks (n=110) were euthanized in pairs by either immersion into 100% CO_2 , or gradual induction to 100% CO_2 at displacement rates of 7, 14, 21 or 28 percentage of chamber volume added per min (%vol/min). CO_2 concentration was measured at chick level. Live focal observations and video recordings were used to assess latency to behavioural responses: head shaking (HS) and gasping (GS) as indicators of distress; loss of posture (LOP) as an indicator of insensibility; and cessation of rhythmic breathing (CRB) and movement (COM), indicating death. All behaviours occurred earliest with immersion compared to gradual treatments, and time between first signs of distress and LOP was shorter for immersion than gradual treatments. Gradual treatments showed a linear decrease in latency to HS, GS and LOP as displacement rate increased. Latency to CRB decreased quadratically with increasing displacement rate, whilst COM decreased linearly. Within gradual treatments, HS and GS occurred at CO_2 concentrations between 0.43-1.14%, LOP between 11.1-17.5%, while CRB and COM occurred between 61.8-78.4%. Overall, immersion induced distress, insensibility and death significantly faster and with the shortest interval between distress and insensibility. For gradual treatment, insensibility and death occurred faster with increasing displacement rates. Behavioural signs of distress were observed with all treatments, and occurred at concentrations lower than those causing insensibility. In conclusion, immersion into 100% CO_2 environment resulted in the shortest time of distress and fastest time to death compared to displacement rates of any speed measured.

4.2 Introduction

Within the poultry industry, cull chicks are routinely euthanized at commercial hatcheries. Maceration, with the use of specialized equipment, has been a commonly used method for humanely killing chicks less than 3 days of age as it results in near instantaneous death via the physical disruption of the brain via fragmentation (Close et al., 1996; Gurung et al., 2018). Although both the Canadian Codes of Practice for Poultry (NFACC, 2016a) and the AVMA (2013) list maceration as an acceptable-with-conditions method of euthanasia for chicks up to 72 hrs, many hatcheries are moving away from maceration for esthetic reasons, and exploring the use of carbon dioxide (CO_2) gas for euthanasia as an alternative.

Gaseous euthanasia involves exposing an animal to high concentrations of an inhalant, resulting in a loss of sensibility and eventual death (Galvin et al., 2005; Raj et al., 2006; AVMA, 2013). Inhalation of CO_2 gas induces insensibility and death via both hypoxia and hypercapnia (AVMA, 2013; Terlouw et al., 2016a). The increased CO_2 and decreased O_2 levels in respired air associated with hypercapnia (AVMA, 2013; Terlouw et al., 2016a) result in acidification of the blood (Gerritzen et al., 2013; Cors et al., 2015; Terlouw et al., 2016a). The reduction in blood pH causes acidification of the cerebral spinal fluid and brain cells, which depressed brain activity, resulting in insensibility, loss of respiratory and cardiac function, and finally death (Gerritzen et al., 2013; Cors et al., 2015; Terlouw et al., 2016a).

Although CO_2 is successful at inducing both insensibility and death, the loss of consciousness is not immediate. There is a period of time between initial exposure to the gas and insensibility that may result in distress (Raj et al., 2006; Gerritzen et al., 2013). CO_2 gas is highly acidic, and forms carbonic acid when in contact with mucosal tissue, which likely causes pain and discomfort (Lambooij et al., 1999; Hawkins et al., 2006; Turner et al., 2012). Furthermore, the depression of the respiratory system by CO_2 results in dyspnea. Dyspnea or breathlessness is the sensation of difficulty breathing or breathing discomfort, which likely is unpleasant and possibly distressing (Hawkins et al., 2006; Raj et al., 2006). Research in rodents indicated it may be possible to induce insensibility at CO_2 levels below the aversive level with certain induction techniques (Burkholder et al., 2010; AVMA, 2013). Similar research with adult poultry has shown conflicting evidence, with some, but not all, suggesting the anesthetic effect of CO_2 may occur prior to it becoming painful (Webster and Fletcher, 2001; Gerritzen et al., 2004; McKeegan et al., 2006).

Gaseous euthanasia of adult birds can be performed by either gradually filling the euthanasia chamber, or by immersing birds into a pre-filled chamber (Close et al., 1996; Galvin et al., 2005). Both methods have their advantages and disadvantages. The gradual addition of 100% CO_2 into the induction chamber is recommended by the AVMA (2013), as it is thought to result in a death involving little pain or distress (AVMA, 2013). This assumption is based on research suggesting a gradual flow rate allows the anaesthetic effect of CO_2 to occur before the gas reaches distressful or painful levels (Gerritzen et al., 2004). This anaesthetic effect is achieved at approximately 17% CO_2 (Gerritzen et al., 2004), whilst indicators of distress are noted at concentrations of 10 to 25% CO_2 (McKeegan et al., 2006), suggesting the loss of consciousness could occur prior to the onset of distress.

The second method, in which the animal is immersed into a pre-filled gas chamber is less documented, but research has shown it induces a rapid loss of sensibility and death, and is faster than the gradual method (Hawkins et al., 2006). A concern with immersion induction is that the rapid transition into high CO_2 atmosphere means the bird is immediately exposed to CO_2 concentrations that may cause pain or distress instead of undergoing a more gradual exposure. Immersion is stated to only be acceptable at low CO_2 concentrations as then it would not cause distress (AVMA, 2013). The consensus in the literature for adult birds is that a gentle death or death with minimal pain that takes longer is preferred to a quick death with obvious signs of distress (Coenen et al., 2000; AVMA, 2013).

Insensibility is a crucial component of euthanasia, as a lack of sensibility means an inability for the bird to feel painful stimuli. The aversive nature of CO_2 means a rapid onset of insensibility is even more vital. Previous studies into gaseous euthanasia during depopulation of poultry have used a loss of posture, defined as when the bird is unable to maintain its upright position and loses neck tension, as an indicator of insensibility (Gerritzen et al., 2004; Gerritzen et al., 2007). A commonly utilized method for indicating loss of sensibility is the loss of somatosensory evoked potentials (SEP) when measuring brain activity by EEG. Research comparing loss of posture to the loss of SEP has shown the former is an indicator of insensibility (Raj, 1998; Gerritzen et al., 2004), suggesting that this measure (loss of posture) indicates loss of sensibility during exposure to CO_2.

There is currently little information available on the proper method of using CO_2 to humanely kill day-old cull chicks. Day-old chicks are more resistant to hypercapnia and anoxia,

as the *in ovo* environment can have CO_2 concentrations as high as 14% (Jaksch, 1981) and arterial pCO_2 in the embryo can reach 60mmHg (Freeman and Vince, 1974). Thus, neonates may be able to tolerate higher concentrations of CO_2 (Jaksch, 1981). This tolerance means data pertaining to adult chickens euthanized by CO_2 may not be transferable, and CO_2 concentrations may need to be higher for chicks and poults compared to older poultry (Jaksch, 1981). A study with neonates in which gaseous euthanasia was conducted via immersion into 60% CO_2 in residual air found that the time until death was longer for neonates than adult birds (Raj et al., 1992), whilst a second study using immersion into 90% CO_2 with residual air concluded that higher concentrations were required for neonates (Raj and Whittington, 1995). This suggests that when using gaseous euthanasia for neonates, a higher CO_2 concentration and a longer exposure time are needed then would be necessary for adult birds.

Recent research by Gurung and colleagues (2018) investigating euthanasia methods for neonates compared gaseous euthanasia via gradual induction with CO_2 or N_2 and euthanasia via low atmospheric pressure stunning. The shortest latencies to insensibility and death were seen with the CO_2 treatments. These treatments were gradual inductions, and 100% CO_2 was introduced continually until final chamber concentrations of 75% or 90% were reached. The authors found no difference in stress response among treatments, and concluded that gradual induction to a final concentration of 75% CO_2 is sufficient for humane euthanasia for male layer neonates (Gurung et al., 2018). The current hatchery practice for the euthanasia of cull poultry with CO_2, involves exposing chicks to CO_2 concentrations of 60 to 70% for 5 minutes (AVMA, 2002; Hester, 2005). However, there is variation in the recommendations made in the literature regarding the concentration, exposure time and displacement or flow rate. To the authors' knowledge, there are no studies of behaviours indicative of distress in neonates. To fully understand the welfare impact of CO_2 gaseous euthanasia on day-old chicks, research is needed to establish the appropriate gas concentrations, displacement rates and exposure times for a rapid onset of insensibility and death, as well as to determine whether CO_2 causes distress for day-old chicks.

The objective of this study was to examine the efficacy of five CO_2 induction methods for euthanizing day-old chicks, including immersion into 100% CO_2 and 4 different displacement rates, on the time to insensibility and death.

4.3 Materials and methods

4.3.1 Animals

All research was approved by the University of Saskatchewan's Animal Care Committee, and by the recommendations of the Canadian Council of Animal Care (1993, 2009). The study was conducted using a total of 126 mixed sex Ross 308 cull broiler chicks from a local commercial hatchery. Cull birds were used as these are most representative of birds requiring euthanasia in industry practice. Experiments were conducted on the day of hatch, and birds were provided with water and feed, and held at 32°C prior to euthanasia to maintain chicks on day of trial.

4.3.2 Experimental design

Carbon dioxide gas induction treatments were evaluated in this experiment in three phases; treatment characterization, a pilot study and a comprehensive replicated experiment. The induction treatments are described in Table 4.1.

Table 4. 1. The five induction treatments used in the study, describing each in terms of the induction type, flow rate and displacement rate

Treatment	Induction type	Flow rate (L/min)	Target Displacement rate (% volume of chamber added/min)
7	Gradual	3	7
14	Gradual	6	14
21	Gradual	9	21
28	Gradual	12	28
100	Immersion	pre-fill	pre-fill

4.3.2.1 Phase I - Treatment characterization

The four gradual CO_2 induction treatments (7, 14, 21 and 28 % vol/min) were characterized to establish the effect of varying displacement rates on the change in CO_2 parameters and ambient conditions within the euthanasia chamber.

4.3.2.2 Phase II – Pilot study

The four gradual treatments were tested on a small sample of birds to establish whether the treatments successfully resulted in insensibility and death, and whether they were appropriate for the euthanasia of day-old chicks and further study. Sixteen cull broiler chicks were obtained from a commercial hatchery on day of hatch, and randomly assigned one of the four gradual induction treatments. Each of the induction treatments was tested twice, with two birds in the chamber per treatment run. Behavioural indicators of insensibility and death were documented via live observation and video recording for both birds in the chamber for all of the treatment runs. For each displacement rate, the maximum and minimum time from gas introduction to insensibility and death were recorded, as well as the overall minimum exposure time required.

4.3.2.3 Phase III – Replicated study

A total of 110 cull broiler chicks were exposed to treatment on day of hatch. The experiment was performed over three days, and day-of-hatch chicks were obtained from a commercial hatchery and exposed to CO_2 on the same day. The five treatments tested were: immersion into 100% CO_2, gradual induction of 100% CO_2 at a displacement rate of 7, 14, 21 or 28 % vol/min. Each treatment was tested 11 times (determined as appropriate with the use of a RCBD power test with estimated means and standard deviations reported in Raj and Gregory, 1990), with each run of the 5 treatments treated as a block. The treatment order within each block was randomized using a computer-generated random number table, and chicks were randomly assigned a treatment. The times from gas introduction until performance of behavioural indicators of insensibility, death, and distress were recorded as well as the CO_2 concentrations at which the behaviours were observed.

4.3.3 Experimental apparatus and procedure

Gas euthanasia was conducted in a 42.5-L Euthanex EZ-197 Induction chamber (Euthanex Corp., Palmer, PA, USA; dimensions: 46×30.5×30.5 cm) via a EP-1305 LPM CO2 Regulator (Euthanex Corp., Palmer, PA, USA) using 99.998% Research Grade CO_2 (Praxair Canada Inc., Calgary, AB, CA). The concentration of CO_2 at chick level was measured using a CM-0121 COZIR Wide range 100% CO_2 Sensor (CO2meter.com, Inc., Ormond Beach, FL,

USA) and monitored with associated GasLab® software (CO2meter.com, Inc., Ormond Beach, FL, USA).

In phase I, the CM-0121 CO_2 sensor was placed at a height of 5.5 to 6 cm in the centre of the chamber wall, opposite to the wall in which the gas inlet was positioned, to monitor the level of CO_2 at chick height. The relative humidity and temperature were assessed using iButton Hygrochron™ DS1923-F5# data loggers (Maxim Integrated; San Jose, CA, USA) at three different locations: centre of the chamber floor, a height of 5 cm from the chamber floor on the chamber wall with the gas inlet, and at 10 cm from the chamber floor on the wall opposite the gas inlet (Figure 4.1). The recording of CO_2 concentration, temperature and relative humidity started two minutes prior to initiation of gas induction to allow for baseline atmospheric recordings.

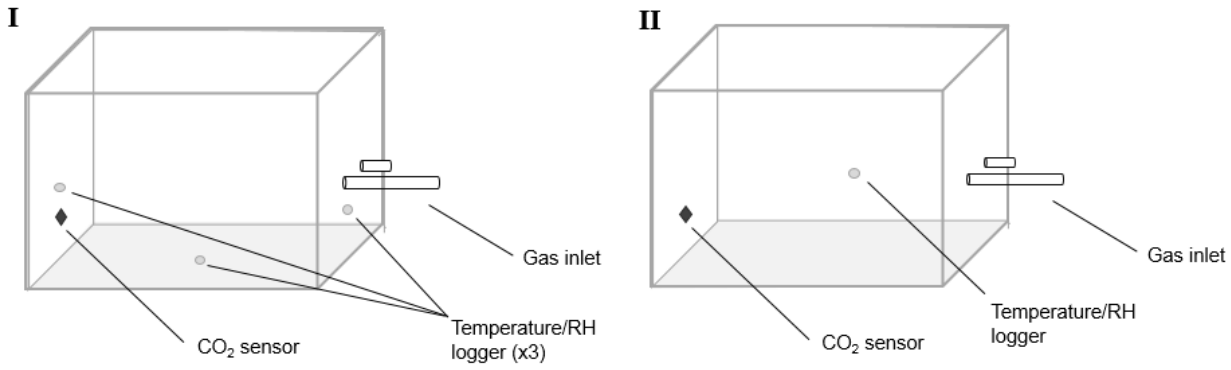

Figure 4. 1. Diagrammatic representation of the experimental apparatus set up, showing the placement of CO_2 sensor and temperature & relative humidity data logger in respect to the location of the gas inlet, for phase I (I) and phases II and III (II) of the experiment.

For phases II and III, the live bird experiments, the CO_2 sensor was placed at a height of 5.5 cm above the chamber floor on the chamber wall parallel to the gas inlet, and the iButton Hygrochron™ DS1923-F5# data logger (Maxim Integrated; San Jose, CA, USA) was placed on the chamber wall adjacent to the gas inlet, to allow for a reading of the environment at chick level without hindering live or video observation. Chamber temperatures consistently remained within 19 to 24°C for the duration of each test. Prior to each treatment, two chicks were weighed and small ink mark made on the head, chest and back of bird one for identification. For gradual induction treatments, the chicks were placed in the chamber and allowed to habituate to the

chamber for one minute, during which baseline atmospheric recordings were taken continuously at a sampling frequency of once per five seconds. The CO_2 gas was then introduced at the appropriate treatment level, and the chick behaviours and reflexes were recorded via live observation and via video cameras (Canon Vixia HFR700 Camcorders; Canon Canada, Mississauga, ON, CAN). The gas flow was maintained until one minute after complete cessation of movement. The birds were then removed from the chamber, cessation of heartbeat was confirmed by stethoscope (Littmann Classic, 3M; London, ON, Canada) and insensibility was confirmed via a lack of response to the pedal reflex withdrawal test (Erasmus et al., 2010). Both chicks were monitored for 5 minutes, after which death was confirmed via both the absence of heartbeat and pedal reflex. For the immersion treatments, the chamber was filled until it reached a concentration of 100% CO_2, after which the birds were placed inside the chamber and the same experimental procedure was followed. For all chicks, cervical dislocation was performed as a secondary euthanasia method, after the 5-minute monitoring period.

4.3.4 Data collection

4.3.4.1 Behavioural observations

Behavioural observation was conducted via focal sampling, with the performance of behaviours recorded continuously from time of gas introduction to cessation of movement, and for both birds in the chamber individually. All live behavioural observations were conducted by two observers throughout the experiment. During live observation, the blinding of observers was not possible, but observation of video recordings were blinded. Reliability between observers was measured and a Pearson correlation coefficient of 0.99 (P<0.01) was found. The behavioural responses of the chicks were used to measure latency to signs of distress from the CO_2, onset of insensibility and death. Head shaking and gasping were considered to be signs of distress (Lambooij et al., 1999; Gerritzen et al., 2004). Loss of posture was used as an indicator of insensibility. The onset of death was split into the respiratory arrest and death. Absence of rhythmic breathing was the indicator used for respiratory arrest, and cessation of movement was used to indicate death. Latency to occurrence of behaviour were recorded as time from introduction of CO_2 to performance of behaviour. Definitions of the behaviours are shown in Table 4.2.

Video recordings were taken with three video camcorders and stored on memory cards (Canon Canada, Mississauga, ON, CAN). The cameras recorded continuously throughout all runs during both phase II and III, and captured the entire chamber. The three cameras captured two perpendicular side views and a top view to ensure different angles and views were available if a bird was not visible in one video. The video recordings were used to verify the live behavioural observation, when the time could not be recorded for an observed behaviour or a behaviour was missed during live observations.

Table 4. 2. Ethogram for behavioural indicators of distress, insensibility and death for day old broiler chicks.

Measure	**Description**
Gasping	Deep breaths with open mouth and out of sync with normal breathing rhythm
Head shaking	Vigorous side to side movement of head and stretched neck
Loss of posture	Inability to remain in initial upright posture combined with a visual loss of neck tension
Rhythmic breathing	The rhythmic movement up and out of rib cage and keel associated with expansion for inhalation, followed by movement of keel and rib cage back down with exhalation. Movement may slow with insensibility, but should remain rhythmic with a consistent time between breathes with a maximum of 3-4 seconds between two breaths.
Cessation of movement	Complete absence of all movement for a minimum of 1 minute.

Adapted from Lambooij et al., 1999; Webster & Fletcher, 2001; Gerritzen et al., 2004 and Erasmus et al., 2010.

4.3.4.2 Carbon dioxide, temperature, and relative humidity collection

During treatment characterization (phase I), CO_2 concentration was continuously measured, then analysed for rate of concentration change, time taken to reach 20 and 40% CO_2, and the maximum concentration CO_2 achieved in the chamber as well as the time taken to reach maximum concentration for each displacement rate. Temperature and relative humidity were measured prior to, during and after the CO_2 induction for each treatment, and analysed for their minimum and maximum values.

For the pilot study (phase II), the CO_2 concentration, temperature and relative humidity within the chamber were measured, and analysed for the minimum and maximum concentrations

required for loss of posture, cessation of rhythmic breathing and movement of the chicks within each gradual treatment, as well as the maximum and minimum temperature and relative humidity reached throughout the experiment.

In the comprehensive study (phase III), each of the treatments was analysed for the CO_2 concentrations at which loss of posture, the cessation of movement and rhythmic breathing occurred, as well as concentration at first performance of behaviours indicative of distress (head shaking and gasping). Temperature and RH were monitored to allow characterization of the environment within the chamber throughout the experiment.

4.3.5 Statistical analyses

4.3.5.1 Phase I

Data for maximum CO_2 concentration and rate of concentration change in phase I was analysed using induction treatment as the main effect via PROC MIXED in SAS 9.4 (SAS Inst. Inc., Cary, NC, USA). When differences were significant, a Tukey-Kramer post hoc test was used for means separation. Regression analyses were performed to determine the relationship between induction method and time to reach maximum concentration and rate of concentration (PROC REG to test linear trends and PROC RSREG for quadratic trends).

4.3.5.2 Phase III

Prior to data analyses, latencies for behavioural observation for each bird were averaged for the two observers and the means from both birds within a chamber were pooled. Birds that recovered were removed from the data set in order to prevent skewing. Behavioural and CO_2 concentration data in phase III were tested for normality and then (log+1) transformed prior to analyses. Main effect of induction treatment (one-way factorial arrangement) was analysed as a randomized complete block design using PROC MIXED, with block as random variable and chamber run as experimental unit. If means were significantly different, means separation was performed using a Tukey-Kramer post hoc test. Regression analyses were performed for gradual induction treatments to determine the effect of displacement rate with PROC REG and RSREG. Multivariate analysis of variance was performed to determine presence of correlation between behaviour and concentration data using PROC GLM. Differences were considered significant when $P \leq 0.05$.

4.4 Results

4.4.1 Treatment characterization

The four induction treatments (7, 14, 21 and 28 % vol/min) all reached a maximum concentration of 100% CO_2 and did so in a quadratic fashion, with time to reach maximum concentration decreasing as displacement rate increased (Table 4.3). A quadratic relationship was also found for CO_2 concentration as a function of time (Table 4.3), with concentration increasing as displacement rates increased.

For all induction treatments, the temperature stayed consistent during the testing, ranging from 19.1 to 20.1°C within the chamber (Table 4.3).

4.4.2 Pilot study

All treatments were capable of inducing insensibility and death, with loss of posture, cessation of rhythmic breathing and cessation of movement occurring for all four treatments. The minimum and maximum latency to onset of behaviour are shown in Table 4.4. The overall minimum and maximum time from gas introduction to loss of posture were 47 and 118 s, respectively, whereas the minimum times for cessation of rhythmic breathing and movement were 141 s and 146 s, respectively, and the maximum times were 543 and 586 s, respectively. The maximum time to insensibility was 118 s (1:58 min) and maximum time to death was 568 s (9:46 min), so the minimum exposure time was established as being 10 minutes.

4.4.3 Definitive study

4.4.3.1 Behaviour

All behavioural indicators had a significantly shorter latency to onset with immersion into a 100% CO_2 chamber compared to when displacement rates of 7, 14, 21 and 24 % vol/min were used (Figure 4.2). Latency to headshaking and gasping for immersion were 1 and 3s, respectively, with latency to loss of posture, cessation of rhythmic breathing and movement being 9, 18 and 56 s, respectively.

Within the gradual induction techniques, faster rates resulted in shorter times (linear) to performance of headshaking, gasping and loss of posture (Table 4.5). Time to cessation of rhythmic breathing had a quadratic relationship with rates, and decreased as rates increased, while cessation of movement decreased linearly.

Table 4.6 presents the time between first performance of distress behaviour and loss of posture, which represents the time in which birds may be conscious to the distressful effects of CO_2. The time between first performance headshaking or gasping, and loss of posture was significantly shorter for the immersion treatment as compared to any of the gradual treatments. Within the latter group, these times were longer when a slow displacement rate of 7 % vol/min was used.

Headshaking and gasping behaviours occurred repeatedly prior to cessation, and both the duration from first to final performance, and the number of incidences of behaviour performance, were less for the immersion treatment than any of the gradual treatments (Table 4.7). The proportion of total time spent performing headshaking was longest for gradual treatment 28 % vol/min, which differed from gradual treatments 7 and 14 % vol/min. Whilst the proportion of total time spent gasping was also longest for the gradual treatment of 28 %vol/min, and differed from the gradual treatment of 7 %vol/min.

4.4.3.2 CO_2 Concentrations

Regardless of the displacement rate, chicks began headshaking, gasping and demonstrated loss of posture at similar CO_2 concentrations (%) (Table 4.8). A difference was noted between treatments for the concentration at which cessation of rhythmic breathing and movement occurred. Rhythmic breathing ceased at a lower concentration of 61.8% with 7 % vol/min compared to 66.8% with 28% vol/min. Cessation of movement occurred at lower concentration for 7% vol/min, then for both 21and 28 % vol/min. Overall the mean concentration for behaviours indicative of distress was between 0.4-1.1%, while insensibility occurred between 11.1–17.5% and death occurred between 70.3-78.4%.

Latency to observed behaviours was positively correlated with the concentration at performance for loss of posture, cessation of rhythmic breathing and cessation of movement. Loss of posture showed a moderate positive correlation, with a correlation coefficient of 0.366 ($P = 0.0187$), whereas cessation of rhythmic breathing and cessation of movement, showed strong positive correlation with correlation coefficients of 0.786 ($P<0.000$) and 0.821 ($P<0.0001$), respectively.

Table 4. 3. Effect of four gradual CO_2 induction treatments on the CO_2 concentration, temperature and relative humidity within the gaseous euthanasia chamber.

	Induction method (% vol/min)							
Environmental variable	7	14	21	28	SEM*	*P* value[1]	*P* value[2]	Equation
CO_2								
Maximum concentration (%)	100	100	100	100	0.0	-	-	
Time to maximum (s)	2040[a]	1270[b]	907[c]	735[d]	189.6	<0.01	<0.01	$y = 18.33x^2 - 415.50x + 3101.25$
Temperature(°C)								
Minimum	19.6	19.6	19.1	19.1				
Maximum	20.1	20.1	20.1	20.1				
Relative Humidity (%)								
Minimum	5.4	4.7	4.7	4.7				
Maximum	15.4	20.9	17.5	23.7				

[a-d] Means within a row with different superscripts differ significantly at $P \leq 0.05$.
*SEM = standard error of the mean.
[1] P value for anova [2] P value for regression

Table 4. 4. The minimum and maximum time from gas introduction to performance of behavioural indicators of insensibility and death for four gradual induction treatments in day-old broiler chicks.

	Latency to performance of behaviour (s)									
	Induction Treatment (% vol/min)									
	7		14		21		28		Overall	
Behaviour observed*	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Loss of posture (i)	90	113	68	88	47	118	48	113	47	118
Cessation of rhythmic breathing (d)	353	543	331	362	148	271	141	240	141	543
Cessation of movement (d)	390	568	335	399	238	283	146	252	146	568

* Behavioural indicator of: (i) insensibility, (d) death.

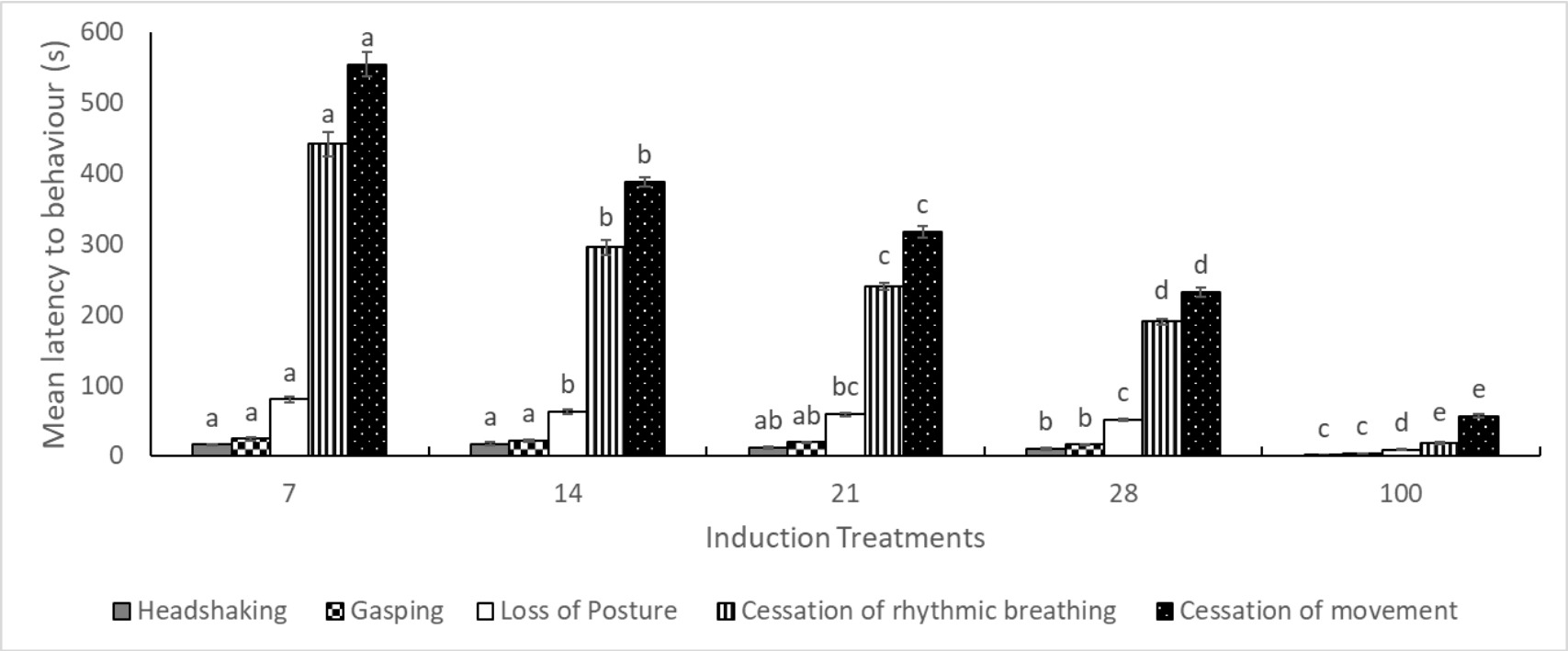

Figure 4. 2. Mean latency to performance of behaviour indicators of distress, insensibility and death by cull broiler chicks in response to four gradual induction treatments of 7, 14, 21 and 28% of chamber volume added per min and one immersion induction into 100% prefill. The behaviours used for distress were headshaking and gasping, insensibility by loss of posture and death were cessation of rhythmic breathing and cessation of movement. Within each behaviour, bars with different superscripts are significantly different ($P \leq 0.05$).

Table 4. 5. Effect of four gradual CO_2 induction treatments on the time from gas introduction to performance of behavioural indicators of distress, insensibility and death in day-old broiler chicks.

Latency to onset of behaviour (s)*	Gradual Induction Treatment (% vol/min)				*P* value[1]	Equation[1]	$R^{2\,(1)}$
	7	14	21	28			
Headshaking (a)	16 ± 1.1[a]	17 ± 2.1[a]	12 ± 1.3[ab]	10 ± 1.0[b]	<0.01	$y = -0.78x + 19.91$	0.31
Gasping (a)	24 ± 1.8[a]	22 ± 1.8[a]	19 ± 1.1[ab]	16 ± 0.9[b]	<0.01	$y = -0.93x + 27.36$	0.24
Loss of posture (i)	80 ± 3.6[a]	62 ± 3.1[b]	59 ± 2.3[bc]	51 ± 2.3[c]	<0.01	$y = -3.01x + 85.54$	0.51
Cessation of rhythmic breathing (d)	441 ± 18.0[a]	295 ± 10.5[b]	240 ± 4.8[c]	190 ± 3.9[d]	0.01	$y = 2.68x^2 - 67.20x + 614.64$	0.87
Cessation of movement (d)	554 ± 17.2[a]	388 ± 6.9[b]	317 ± 7.8[c]	232 ± 6.8[d]	<0.01	$y = -34.61x + 632.36$	0.89

[a-d] Means ± SEM within a row with different superscripts differ significantly at $P \leq 0.05$.

[1] Values for *P*, regression equation and R^2 based on log-transformed values.

* Behavioural indicator of: (a) distress, (i) insensibility, (d) death.

Table 4. 6. Effect of CO_2 induction treatment on the time between performance of behaviours indicative of distress and loss of posture in day old broiler chicks.

	Induction Treatment[1]						
	Gradual (% vol/min)				Immersion		
Time between distress behaviour and insensibility (s)	7	14	21	28	100	*P* value	SEM
Headshaking to loss of posture	64[a]	45 [b]	47[b]	41[b]	8[c]	<0.01	0.101
Gasping to loss of posture	56[a]	40[b]	40[b]	35[b]	6[c]	<0.01	0.107

[a-c] Means within a row with different superscripts differ significantly at $P \leq 0.05$.

[1] n = 11.

Table 4. 7. Effect of CO_2 induction treatment on the duration, incidences, rate and proportion of time spent headshaking and gasping in day old broiler chicks.

	Induction Treatment					
	Gradual (%vol/min)				Immersion	
Behaviour observed	7	14	21	28	100	*P* value
Headshaking						
Duration (s)	26.0±3.54[a]	20.2±1.91[a]	18.6±2.29[a]	17.9±1.95[a]	4.1±0.60[b]	<0.01
Incidence (n)	7.8±0.71[a]	7.0±0.47[a]	6.9±0.61[a]	6.8±0.58[a]	2.6±0.40[b]	<0.01
Proportion of total time	0.05±0.005[c]	0.05±0.005[bc]	0.06±0.007[abc]	0.08±0.010[a]	0.07±0.011[ab]	<0.01
Gasping						
Duration	31.0±4.07[a]	25.7±3.18[a]	27.7±3.84[a]	22.1±2.75[a]	4.1±0.49[b]	<0.01
Incidence	11.3±1.51[a]	9.5±1.35[a]	9.5±1.18[a]	9.6±1.36[a]	3.6±0.33[b]	<0.01
Proportion of total time	0.05±0.090[b]	0.06±0.008[ab]	0.09±0.012[ab]	0.10±0.014[a]	0.07±0.009[ab]	0.01

[a-c] Means within a row with different superscripts differ significantly at $P \leq 0.05$.

Table 4. 8. Effect of four gradual CO_2 induction methods on the CO_2 concentration at which behavioural indicators of distress, insensibility and death are observed in day-old broiler chicks.

	Induction Treatment (% vol/min)[1]					
Behaviour observed *	7	14	21	28	*P* value	SEM
CO_2 concentration at behaviour performance (%)						
Headshaking (a)	0.43	0.91	0.48	0.56	0.77	0.137
Gasping (a)	0.96	0.95	0.89	1.14	0.97	0.159
Loss of posture (i)	11.99	11.13	14.19	17.53	0.59	1.423
Cessation of rhythmic breathing (d)	61.79^{b}	63.28^{ab}	65.80^{ab}	66.75^{a}	0.01	0.657
Cessation of movement (d)	70.27^{b}	73.64^{ab}	78.39^{a}	75.91^{a}	<0.01	0.754

[a-b] Means within a row with different superscripts differ significantly at $P \leq 0.05$.
[1] n = 11.
* Behavioural indicator of: (a) distress, (i) insensibility, (d) death.

4.4.3.3 Unsuccessful Euthanasia

Throughout the experiment there were four birds that regained consciousness within five minutes after removal from the Euthanex box. These occurred with gradual treatments of 7 (1 bird), 21 (1 bird) and 28 (2 birds) % vol/min. No birds regained consciousness when exposed to a 100% prefilled CO_2 chamber.

4.5 Discussion

4.5.1 Insensibility and death

The recommendation for gas euthanasia of older birds involves a fast gas introduction into the euthanasia chamber, with a displacement rate of 10 to 30% of chamber volume added per minute (AVMA, 2013) or a gradual increase by introduction of 100% CO_2 to a minimum concentration of 40% for an exposure time of 30 minutes (Gerritzen et al., 2004). The evidence from this study shows that fast induction rates are not ideal for the euthanasia of day old chicks. In fact, the time to loss of sensibility was more than 80% quicker for chicks placed into a 100% pre-filled environment as compared to the high displacement rate tested (8 s for 100% immersion vs 41 s for gradual treatment of 28% vol/min).

Our findings provide further evidence that there is a difference in response to CO_2 between neonates compared to the published literature for older birds, as the concentrations at which distress, insensibility and death occurs for day-of-hatch broiler chicks in response to CO_2 euthanasia are different from the concentrations shown by research involving older birds. In response to gradual induction of CO_2 to a maximum concentration of 40%, week-old broilers showed head shaking at 8.3% CO_2, gasping at 9.2%, loss of posture at 19% and death at a final concentration of 40% (Gerritzen et al., 2007). Although the concentration at loss of posture occurs is within a similar range to that found in this experiment, the concentration at which death occurs is much lower in older birds, whilst the concentrations for behaviours indicative of distress are much higher (Table 8). Our results suggest that the neonates' higher tolerance to CO_2, previously shown by Raj and colleagues (Raj et al., 1992; Raj and Whittington, 1995), increases time to death and may affect the onset of insensibility. This suggests that chicks do not have an increased tolerance to the distress resulting from CO_2. The tolerance to hypercapnia post hatch has been attributed to the higher CO_2 environment in the egg during incubation (Jaksch, 1981). More specifically, the tolerance may be attributed to the compensatory mechanism utilized by the embryo to counteract the hypercapnic environment and threat of respiratory acidosis (Burggren et al., 2015), resulting from the increased pCO_2 in the blood and air cell during incubation due to the increasing metabolic demands of the embryo (Freeman and Vince, 1974). *In ovo*, chicks regulate the acid-base balance by increasing the bicarbonate concentration (HCO_3^-) to compensate for this increased pCO_2 in the air cell and blood (Burggren et al., 2015). This embryonic compensatory mechanism may still be present in neonates on the day of hatch and thus help explain the higher tolerance of neonate cells to hypercapnic death. As the behaviours indicative of distress involve other physiological processes and responses to CO_2, this mechanism could explain why no increased tolerance was seen for these behaviours.

The consistent variation in time to insensibility and death with the variation in displacement rates noted within the literature suggests the onset of insensibility and death are dependent on the atmospheric and inspired CO_2 concentrations rather than the rate of gas introduction. This is shown in our findings, as the fastest induction was with immersion in which the atmospheric CO_2 concentration is already present and high at chick introduction. In this treatment, the time to insensibility and death were not dependent on the time for atmospheric CO_2 to reach a sufficient concentration, but instead on the time for the body to respond

physiologically to CO_2, for the CO_2 to saturate the tissues and result in depression of neuron function and eventually cell death (Lambooij et al., 1999). The next fastest induction was with the fast displacement rate, as the faster gas introduction meant the chamber reached the necessary concentrations quicker.

4.5.2 Aversion and distress

That distress and aversion occurs with high concentrations of CO_2 and has a negative welfare impact is well-established in mammals. Pain is the most commonly described negative sensory experience and in CO_2 euthanasia, pain is associated with carbonic acid formation on the mucous membranes of the upper respiratory tract (Lambooij et al., 1999; Coenen et al., 2000). Pain has been well documented in humans and other mammals during exposures to high levels of CO_2 on induction, but there is discussion as to whether CO_2 induction is painful in birds (McKeegan et al., 2006; McKeegan et al., 2007). Dyspnea, or breathlessness, is another negative experience commonly associated with distress during CO_2 gas exposure (Gerritzen et al., 2004; Raj et al, 2006; Gerritzen et al., 2007) and can be described in terms of its three different qualities: respiratory effort, air hunger and chest tightness (Beausoleil and Mellor, 2015). An increase in atmospheric CO_2, such as with gaseous euthanasia, will result in both unpleasant respiratory effort and air hunger. Respiratory effort is an awareness to an increased effort needed to achieve the necessary level of ventilation (Beausoleil and Mellor, 2015). During CO_2 euthanasia there will be an unpleasant increase in respiratory effort as lack of available O_2 will cause tissue hypoxia in the respiratory muscle, thus decreasing the muscular functioning and respiratory ability (Beausoleil and Mellor, 2015). Air hunger is the increased urge or increased need to breathe, and occurs with CO_2 euthanasia as the increased arterial pCO_2 resulting from hypercapnia increases the automatic command to breathe, and results in air hunger (Raj et al., 2006; Beausoleil and Mellor, 2015). Breathlessness has also been shown to activate the same regions of the brain as other negative sensory experiences such as pain, hunger and thirst in mammals (Beausoleil and Mellor, 2015), suggesting that the dyspnea occurring with CO_2 is a significant stressor and an important factor in the welfare impact of gaseous euthanasia. Gasping is an indicator of respiratory distress and breathlessness (Gerritzen et al., 2004; Raj et al., 2006), as the act of gasping implies an increased effort needed to breathe (Coenen et al., 2000), and is

mediated by receptors in the lower respiratory tract that respond to a slight increase in inspired CO_2 concentration (McKeegan et al., 2005).

Head shaking has been proposed as being either an indication of irritation of gustatory or nasal trigeminal receptors (McKeegan et al., 2006) or an indication that birds are dizzy or disorientated (Gerritzen et al., 2007), thus indicative of negative subjective experiences of either pain or disorientation. Despite not being able to quantify the experience of distress, the observation of headshaking and gasping with all of the induction treatments suggests that CO_2 is distressful to neonates whether this be due to pain, breathlessness, or another unknown negative sensory experience.

Recommendations for euthanasia with CO_2 for adult birds state that a mild or non-distressful slow death is preferable to a rapid death with clear indications of distress (Coenen et al., 2000; AVMA, 2013), based on the assumption that a slower death minimizes the distress associated with CO_2 and allows for onset of anaesthetic effect of CO_2 prior to pain or distress. Our study showed that in day old chicks, behaviours indicative of distress occurred for all induction treatments, regardless of speed of introduction of gas or immersion. Headshaking and gasping also occurred at concentrations much lower than the concentration at which loss of posture occurred. This difference in CO_2 concentration at which behaviours occur (as well as the latency to performance of behaviours), means that the duration of time between first behavioural indication of distress and loss of posture is a period prior to insensibility in which the chicks are conscious and could experience pain and discomfort. Behaviours indicative of distress are also performed repetitively and continually until loss of posture, giving further indication that this entire period is distressful (Gerritzen et al., 2007). Although our findings showed a higher proportion of time spent performing distress behaviours with the faster gradual treatment, this is likely to be due to the significantly shorter latencies to death rather than performance of behaviours themselves, as the duration of this were equal. Thus an induction method with the shortest duration of time spent performing indicators of distress and with the shortest time between first indicators of distress and loss of posture have the least negative welfare impact. Based on the behavioural responses seen with all induction treatments, it can be concluded that a gentle distress-less death with CO_2 did not occur. Thus the faster insensibility occurs, the less time neonates are conscious to the breathlessness, disorientation, pain and possible aversion.

4.6 Conclusion

From this experiment it can be concluded that when comparing the efficacy of CO_2 induction treatments for neonates, immersion into a euthanasia chamber pre-filled to 100% CO_2 induced a rapid loss of consciousness and death, significantly faster than any gradual induction treatments. The immersion treatments also had the shortest period of time between first indicators of distress and insensibility, thus the least amount of time the chick can perceive negative sensory aspects of CO_2 that result in distress. With the gradual induction method, the faster rate of CO_2 introduction was associated with shorter latencies to insensibility and death. Behavioural indicators of distress were observed with all the treatments, and started at low CO_2 concentrations (<1.5%). This observation demonstrates that with neonates there is no opportunity for anesthetic effect of CO_2 to occur prior to distress and there is no increased tolerance to the distressful components of CO_2, thus a slow gentle death does not seem to occur. Therefore, euthanasia via an induction treatment with a shorter exposure to the distressful conditions of CO_2 may be the most welfare friendly for chicks on day of hatch. The immersion of chicks in 100% CO_2 in a commercial hatchery may be difficult to achieve, thus at present research is ongoing to investigate euthanasia via immersion into lower concentrations of CO_2.

4.7 Acknowledgements

The author would like to thank Prairie Pride Hatchery for the donated cull chicks, as well as Chicken Farmers of Saskatchewan, Poultry Industry Council, Canadian Poultry Research Council, Egg Farmers of Canada, Hybrid Turkeys, the Ontario Ministry of Agriculture, Food and Rural Affairs, and Natural Sciences and Engineering Research Council for funding this study.

5.0 Chapter Five - Defining characteristics of immersion CO_2 gas for successful euthanasia via immersion of neonates and young broilers.

Research presented in Chapter 4 examined different CO_2 induction methods and established that gaseous euthanasia via immersion into 100% CO_2 was the most efficacious CO_2 induction method with the shortest duration of distress. However, in commercial hatcheries, a concentration of 100% CO_2 might be difficult to achieve, so research is required to elucidate how immersion into different concentrations of CO_2 affects the efficacy and welfare impact of immersion induction is required. The objective of research in Chapter 5 was to ascertain how four different CO_2 concentrations affect the efficacy and welfare impact of gaseous euthanasia and to evaluate how the efficacy of CO_2 induction methods change with age, by measuring behaviour and blood gas and chemistry parameters.

5.1 Abstract

Immersion induction with a chamber pre-filled to a concentration of 100% CO_2 is an effective method for the euthanasia of cull chicks on day of hatch. However, 100% CO_2 might be difficult to achieve in commercial hatcheries. This study was conducted to elucidate how varying CO_2 concentrations within the euthanasia chamber affect the efficacy of CO_2 immersion euthanasia in regards to distress, insensibility and death, and, to evaluate how the efficacy of CO_2 induction methods change with bird age. Five treatments were evaluated for their effect on distress, insensibility and death; four immersion inductions with immersions into concentrations of 70, 80, 90 or 100% CO_2, and a gradual induction with a displacement rate of 28% of chamber volume added per minute. In the first experiment, broiler chicks (n=192) at 0, 3 and 6 days of age were immersed, in pairs, into one of the four CO_2 concentrations. For the second experiment, chicks (n=88) at 3 and 6 days of age were euthanized, in pairs, by either immersion induction with 100% CO_2 or gradual induction. Behavioural observations were performed via live focal sampling and video recordings to measure latency to behavioural response: loss of posture (LOP) as an indicator of insensibility, and cessation of rhythmic breathing (CRB) and cessation of movement (COM) as indicators of death. The latency to performance, duration, frequency and proportion of total time spent performing specific activity were measured for the behaviours indicative of distress; headshaking (HS) and gasping (GS). Data for both experiments were analysed for effects of CO_2 method and age (two-way factorial), as an RCBD, with each round of treatments as a block. Regression analyses were performed on behavioural data from experiment one for the effect CO_2 concentration had on latency to behaviour. The concentration of CO_2 used for immersion had no effect on the latency to performance of HS, GS and LOP, but for both CRB and COM, immersion into 90 % and 100% resulted in shorter latencies than immersion into 80%, and all three concentrations resulted in shorter latencies than immersion into 70%. Age decreased the latency to CRB and COM, with the longest latencies seen for chicks at 0 days. Gradual induction resulted in the longest latencies to HS, GS, LOP, CRB and COM, and the longest duration and highest frequency of HS and GS, at all ages. Induction via immersion into 100% induced insensibility and death quickest, with a short duration and low frequency of distress behaviour performance. Immersion into 90% CO_2 result in latencies to distress, insensibility and death equivalent to those of immersion into 100% CO_2. For chicks at 0 days of age, the time to death was longer for immersion into 70% than previously seen with gradual

induction treatment for 0 day old chicks. Time to death decreases as birds age. In conclusion, immersion of chicks into 90 or 100% CO_2 causes a rapid onset of insensibility and death, with short duration of distress.

5.2 Introduction

Gaseous euthanasia, or the exposure of an animal to high concentrations of inhalant that will lead to insensibility followed by death (Galvin et al., 2005; Raj et al., 2006; AVMA, 2013; Baker et al., 2019), is a method of euthanasia that is currently being used for culling both adult and neonate birds. Carbon dioxide (CO_2) is one of the most common and widely utilized inhalants for gaseous euthanasia (HSA, 2006; AVMA, 2013). Insensibility and death occur with CO_2 inhalation as a result of both hypercapnia, as the concentration of CO_2 increases, and hypoxia, as the availability of oxygen decreases (AVMA, 2013; Terlouw et al., 2016a). Excessive CO_2 inhalation, which occurs with gaseous euthanasia, elevates the level of CO_2 in the blood (hypercapnia), causing a rise in carbonic acid and the concentration of H^+ ions in the blood, decreasing the pH and resulting in acidosis. The normal pH value of the blood for chickens is around 7.4 to 7.5 (Guo et al., 2008; Martin et al., 2010; Montesinos and Ardiaca, 2013; Reece, 2015b; Schaal et al., 2016). The level of CO_2 in the blood is often described via the partial pressure of CO_2 ($pCO2$), which is a measure of the tension or pressure of CO_2 dissolved in the blood. It describes the balance between CO_2 produced by the cells and CO_2 lost via respiration (Reece, 2015b). A rise in pCO_2 above 42mm hg is considered hypercapnia, and a pCO_2 above 45mm hg can result in hypercapnic respiratory failure. In response to hypercapnia and the corresponding respiratory acidosis, the respiration rate will increase in order to clear more CO_2 via ventilation and return to equilibrium (Gerritzen et al., 2006; Reece, 2015b). With gaseous euthanasia, the elevated CO_2 in inhaled air means the increased respiration rate will not be able to correct the imbalance but instead will exacerbate it, resulting in a rise of pCO2 and a drop in pH (Forslid and Augustinsson, 1988; Gerritzen et al., 2006). The drop in pH causes acidosis of the cerebral spinal fluid, leading to both respiratory depression (Terlouw et al., 2016a) and the acidification of brain cells, depressing brain activity (Gerritzen et al., 2013; Cors et al., 2015; Terlouw et al., 2016a). Consciousness is lost as a consequence of the depression of brain activity, and the further reduction in brain and respiratory activity leads to a total loss of brain, respiratory and cardiac function, followed by death (Gerritzen et al., 2013; Cors et al., 2015; Terlouw et al., 2016a). The occurrence of hypercapnia coincides with the occurrence of hypoxia, as the increase in atmospheric CO_2 displaces O_2, meaning there is less O_2 available in the respired air and in the bloodstream. The partial pressure of O_2 ($pO2$), is often used to describe the O_2 levels in the blood, and a $pO2$ below 80mmhg is considered hypoxia. The lack of

O_2 in the bloodstream means there is insufficient O_2 available for cellular energy metabolism; as a result, cells will undergo metabolic crisis and cell death occurs (ESFA, 2004). The occurrence of extensive cell death will render the brain, cardiovascular and respiratory systems non-functional, resulting in death (ESFA, 2004).

The acidic nature of CO_2 means that when it comes into contact with the mucosal tissues, it forms carbonic acid (Lambooij et al., 1999; Hawkins et al., 2006; Turner et al., 2012). This is a possible welfare concern as it could cause the bird pain and distress (Lambooij et al., 1999; Hawkins et al., 2006; Turner et al., 2012). Furthermore, the respiratory depression that occurs with CO_2 euthanasia causes a sensation of having breathing discomfort or difficulty. This sensation, known as dyspnoea or breathlessness, is likely to be unpleasant and distressful (Hawkins et al., 2006; Raj et al., 2006). The occurrence of distress from CO_2 inhalation is a concern for its use in gaseous euthanasia, as the induction of insensibility with CO_2 is not instantaneous, meaning there is a time between the start of CO_2 exposure to when insensibility occurs during which birds may be conscious to the distress associated with CO_2 (Raj et al., 2006; Gerritzen et al., 2013; Baker et al., 2019). Minimising the time between initial exposure and loss of consciousness would then minimise some of the distress and suffering associated with CO_2 euthanasia.

A number of methods have been studied with respect to euthanasia of neonate poultry, including CO_2 (Gurung et al., 2018; Baker et al., 2019). Recent research by Gurung and colleagues (2018) concluded that euthanasia with CO_2 had the shortest time to insensibility and death compared to euthanasia via N_2 inhalation or Low Atmospheric Pressure Stunning. These authors also reported that stress levels, as measured by serum corticosterone and serotonin concentrations, from the three methodologies were similar. Further research into the CO_2 euthanasia of day-old chicks by Baker and colleagues (2019) investigated the efficacy of different induction methods and the associated distress. The study found that immersion into a chamber pre-filled to 100% CO_2 resulted in the shortest time during which animals were conscious to the possible distressful concentrations of CO_2, the shortest duration and lowest frequency of performance of behaviours indicative of distress, as well as the shortest time to insensibility and death compared to gradual induction of the gas (Baker et al., 2019).

The current recommendations for the gaseous euthanasia of adult birds suggest the use of slow, gradual induction (Coenen et al., 2000; AVMA, 2013), as a slower induction is assumed to reduce the distress resulting from CO_2 exposure and to inhibit consciousness prior to the onset of distress. Neonate chicks have been shown to perform behavioural indicators of distress at CO_2 concentrations well below (<1.5%) those at which consciousness is lost (12-18%) and they perform these continuously and repetitively until insensibility occurs (Baker et al., 2019). This suggests that the entire period between the start of the expression of distress behaviours until insensibility occurs is stressful for birds (Baker et al., 2019). The difference between neonate response to CO_2 euthanasia and that of adult poultry may be attributed to the higher tolerance of day-old chicks to CO_2 and hypercapnia (Jaksch, 1981; Raj et al., 1992), with previous studies showing that both longer exposure times and higher CO_2 concentrations are required in the euthanasia of neonatal birds (Raj et al., 1992; Raj and Whittington, 1995). There is little scientific understanding of how the CO_2 tolerance changes with age and how this change affects the level of distress experienced by the birds. Gaining an understanding of this is vital to be able to fully understand the welfare impact of gaseous euthanasia for young birds and to be able to develop recommendations regarding the use of CO_2 euthanasia techniques.

Research by Baker et al. (2019) compared CO_2 euthanasia via either gradual induction (birds are placed into the euthanasia chamber and then CO_2 is gradually introduced into the chamber) or pre-fill immersion (the chamber is pre-filled to a certain CO_2 concentration and the birds are then placed into the pre-filled chamber) induction. They concluded that for the euthanasia of chicks on day-of-hatch, immersion into a chamber pre-filled to 100% CO_2 is the most welfare friendly induction method of those compared. Within a farm or hatchery setting, it might not always be possible to ensure that the birds can be immersed into 100% CO_2, as the source of CO_2 cannot be guaranteed to be 100%, or that the setup of the equipment does not allow for it. It is not known how a different CO_2 concentration in the chamber effects the efficacy of immersion induction for neonate euthanasia. Research has found that with gradual induction, death occurs at concentrations of 70 to 79% with neonates (Baker et al., 2019) and a final concentration of 75% CO_2 is sufficient to result in death (Gurung et al., 2018), while a final concentration of 40% is required for adult birds (Gerritzen et al., 2007). These concentrations pertaining to death with gradual induction are not directly applicable to immersion induction, as the immediate increase in inhaled CO_2 versus the slow build-up of inhaled CO_2 may differ

slightly in physiological response, and thus have different requirements for minimum concentrations. Thus research is required to understand how the concentration into which chicks are immersed affects euthanasia success, as well as whether an effect of different concentrations impacts insensibility and distress associated with CO_2. The objective of this study was to understand the welfare impact of euthanasia via immersion into high concentrations of CO_2 on young birds. The specific objectives were 1) to evaluate the effect of immersion concentration on the efficacy of gaseous euthanasia, and 2) to determine how age affects the efficacy of gaseous euthanasia via either gradual or immersion induction.

5.3 Materials and methods

5.3.1 Animals

All research was approved by the University of Saskatchewan's Animal Care Committee and by the recommendations of the Canadian Council of Animal Care (1993, 2009). The study involved a total of 280 mixed sex Ross 308 broiler chicks from a local commercial hatchery, with 192 of birds being used in Experiment 1 and 88 birds in Experiment 2. The birds were housed under commercial conditions as recommended by the Aviagen management guide and fed a commercial broiler starter diet.

5.3.2 Experimental design

This research evaluating gaseous euthanasia induction methods and age encompassed two experiments; the first investigated the effects of immersions into CO_2 concentration of 70, 80, 90 and 100% CO_2 at 0, 3, and 6 days of age, whilst the second investigated the effect of immersion or gradual induction on birds at 3 and 6 days of age.

5.3.2.1 Experiment one – Immersion concentration

This experiment investigated two main effects – level of CO_2 with immersion into five CO_2 concentrations, either 60, 70, 80, 90 or 100% CO_2, and chick age (0, 3 or 6 days of age). However, in a preliminary experiment used to validate the treatments, the 60% immersion treatment did not result in death within 20 minutes; thus it was removed from the trial, leaving four immersion treatments.

Each remaining treatment was tested 8 times (determined as appropriate with the use of a RCBD power test with estimated means and standard deviations from a previous experiment,

(Baker et al., 2019)), with one run of all 4 treatments used as a block. The treatments within a block were randomised using a computer-generated random number table, and birds were randomly assigned to each treatment.

5.3.2.2 Experiment two – Gradual versus immersion

The second experiment investigated two gaseous euthanasia treatments; either immersion into 100% carbon dioxide or gradual induction with a displacement rate of 28% chamber volume added per minute, at 3 and 6 days of age. Each treatment was tested 11 times (determined as appropriate with the use of a RCBD power test with estimated means and standard deviations from Baker et al., 2019), with each group of treatments used as block. The treatments within a block were randomised using a computer-generated random number table, and birds were randomly assigned to each treatment.

5.3.3 Experimental apparatus and procedure

A 42.5-L Euthanex EZ-197 Induction chamber (Euthanex Corp., Palmer, PA, USA; dimensions: 46×30.5×30.5 cm) with a EP-1305 LPM CO_2 Regulator (Euthanex Corp., Palmer, PA, USA) was used with 99.998% Research Grade CO_2 (Praxair Canada Inc., Calgary, AB, CA) for the gaseous euthanasia. The CO_2 concentration at bird level within the chamber was recorded using a CM-0121 COZIR Wide range 100% CO_2 Sensor (CO2meter.com, Inc., Ormond Beach, FL, USA) and corresponding GasLab® software (CO2meter.com, Inc., Ormond Beach, FL, USA).

The CO_2 sensor was located on the chamber wall parallel to the gas inlet at the height of 5.5 cm, and the iButton Hygrochron™ DS1923-F5# data logger (Maxim Integrated; San Jose, CA, USA) was located on the wall adjacent to the gas inlet, to allow for a reading of the environmental conditions at chick level without hindering live or video observation. Chamber temperatures consistently remained within 22 to 28°C for the duration of both experiments.

Prior to treatment, two broiler chicks were weighed, and bird one was marked for identification with a small ink mark on the head, chest and back. For experiment one, the chamber was filled until it reached the desired treatment concentration of either 70, 80, 90 or 100% CO_2. The birds were then placed into the chamber and behaviours and reflexes performed by the birds were recorded via both live observation and via video cameras (Canon Vixia

HFR700 Camcorders; Canon Canada, Mississauga, ON, CAN). Birds were removed from the chamber one minute after final observed movement. A blood sample was taken from one of the birds, by decapitation, and immediately analysed via the i-Stat 1 (Abbott Point of Care Inc.; Abbott Park, IL, USA). The second bird was evaluated to confirm a cessation of heartbeat via a stethoscope (Littman Classic, 3M; London, ON, Canada) and insensibility via a lack of response to corneal blink reflex and pedal reflex withdrawal test (Erasmus et al., 2010b). The second bird was then monitored for 5 minutes to ensure consciousness did not return after which death was confirmed via both the absence of heartbeat, corneal blink and pedal reflex, and cervical dislocation was performed as a secondary euthanasia method.

For experiment two, the chamber was filled to a concentration of 100% CO_2 for the immersion treatment, after which the bird was placed into the pre-filled chamber. With respect to the gradual induction treatments, birds were placed into the chamber and allowed to habituate for 30 seconds; then CO_2 was introduced into the chamber at a displacement rate of 28% of the chamber volume per minute. The birds inside the chamber were live observed and video recorded throughout the euthanasia process. Once final movement was confirmed both birds were removed from the euthanasia chamber. Both birds were evaluated to confirm insensibility and a cessation of heartbeat. The birds were monitored for 5 minutes to ensure consciousness did not return, after which cervical dislocation was performed as a secondary euthanasia method.

5.3.4 Behavioural observations

Focal sampling was used for live behavioural observation, with the performance of specific behaviours being recorded continuously from the point of gas introduction to removal from the chamber. The behaviours were recorded individually for both birds within the chamber. Two trained observers conducted the observations throughout the experiment, however, as the live observations did not allow for the blinding of observers, the observation of confirmational videos was performed blind to treatment. The behaviour responses measured were those previously used in gaseous euthanasia with CO_2 for neonate poultry (Baker et al., 2019), and included the latency to head shaking and gasping, the latency to loss of posture, the latency to cessation of convulsions and the latency to cessation of rhythmic breathing and total movement. Head shaking and gasping were recorded as behaviours indicating distress in response to CO_2 (Lambooij et al., 1999; Gerritzen et al., 2004). The frequency of behaviour performance, the

duration of performance, and the proportion of conscious time and proportion of total time spent performing distress behaviours were also measured for headshaking and gasping, and loss of posture was the behaviour indicative of insensibility. Cessation of rhythmic breathing, convulsions and total cessation of movement were used as indicators of death. Behaviours were recorded as the latency to occurrence of behaviour from the moment the bird's feet came into contact with the chamber floor for immersion or from introduction of CO_2 into the chamber for gradual treatments until the performance of the behaviour. An ethogram with definitions of the behavioural indicators can be found in Table 5.1.

Behaviours were also recorded via three video camcorders continuously throughout all runs for both experiments The cameras captured the entire chamber, with the three cameras capturing two perpendicular side views and a top view to ensure different angles and views were available if a bird was not visible in one video. The video recordings were used to as a verification for all live behavioural observation.

5.3.5 Blood gas and chemistry parameters

Whole blood was collected from one bird per run in an EDTA tube and then analysed immediately (within 60 seconds) via an i-STAT portable blood analyser with either CG8+ or CG4+ i-STAT cartridge (Abaxis Inc.; Union City, CA, USA). Blood samples were evaluated for the blood gas parameters of blood pH, the partial pressure of CO_2 (*p*CO2) and O_2 (*p*O2) in the blood. The partial pressure reflects the amount of CO_2 or O_2 dissolved in the in the blood. The blood parameter were categorized into ranges and percentage of birds with values within these ranges were calculate, with pH ranges being below 6.50, between 6.50 and 6.69, between 6.70 and 6.89, and equal to or above 6.90; the *p*CO2 ranges included equal to or less than 70, between 71 and 100, between 101 and 130, or above 130; the *p*O2 ranges being between 25 and 50, between 51 and 75, between 76 and 100, between 101 and 125, or above 125.

Table 5. 1. Ethogram for behavioural indicators of distress, insensibility and death.

Measure	Description
Gasping	Deep breaths with open mouth and out of sync with normal breathing rhythm
Head shaking	Vigorous side to side movement of head and stretched neck
Loss of posture	Inability to remain in initial upright posture combined with a visual loss of neck tension
Rhythmic breathing	The rhythmic movement up and out of rib cage and keel associated with expansion for inhalation, followed by movement of keel and rib cage back down with exhalation. Movement may slow with insensibility but should remain rhythmic with a consistent time between breaths with a maximum of 3-4 seconds between two breaths
Convulsions	Uncontrolled involuntary muscle contraction that involves wing-flapping and leg pedalling
Cessation of movement	Complete absence of all movement for a minimum of 1 minute

Adapted from Lambooij et al., 1999; Gerritzen et al., 2004 and Erasmus et al., 2010b.

5.3.6 Statistical analyses

Behavioural observation data for each bird was averaged for the two observers, and the means were pooled from both birds within a chamber. Behavioural data were tested for normality and then (log+1) transformed prior to analyses. For experiment one, the main effects of CO_2 concentration and age (two-way factorial) were analysed as a randomised complete block design using PROC MIXED in SAS 9.4 (SAS Inst. Inc., Cary, NC, USA) for behaviour and blood parameter data, with each run of all treatments as the random variable and chamber run as experimental unit. Regression analyses were performed on the behaviour data in experiment one, to determine the effect of CO_2 treatment by age via PROC REG and RSREG. Behavioural data from experiment two were analysed to determine the effects of CO_2 induction method and age via PROC MIXED, with each group of induction treatments as the random variable and chamber as experimental unit. For both experiments, means separation was performed using a Tukey-Kramer post hoc test when means were significantly different, with differences considered significant when $P \leq 0.05$.

5.4 Results

5.4.1 Experiment one

5.4.1.1 Latency to behaviour observations

A linear decrease occurred for latency to loss of posture as immersion concentration increased at 0 days of age and for cessation of both rhythmic breathing and movement at 6 days of age (Table 5.2). Both behavioural indicators of death demonstrate a positive quadratic relationship with CO_2 concentration at 0 and 3 days of age. Figure 5.1 displays the effect of age on latency to behavioural indicators of death and illustrates that as the birds age they become less tolerant to the CO_2 concentration, with the lower concentration of immersions having less effect on increasing the latency to death.

No effect was noted for either CO_2 treatment or chick age for latency to perform headshaking (Table 5.3). Latency to cessation of rhythmic breathing and movement showed an interaction between age and concentration (Table 5.4 and 5.5). The longest latencies were seen for 70% at day 0, and the shortest times being seen for 100% at day 0 and 80, 90 and 100% at day 3, with the latencies for both behaviours not differing between 90% and 100% regardless of age. It was also noted that the lower the concentration of CO_2 resulted in a longer latency to

performance of behaviours indicative of death, with chicks exposed to 90 and 100% being equal. Latency to gasping and loss of posture differed with age, as chicks 6 days of age performed gasping longer than birds at 3 days of age, and at 6 days of age time to loss of posture was longer than for chicks of both 0 and 3 days of age. For the behavioural indicators of death, the effect of age resulted in a longer latency for 0 day old chicks than both 3 and 6 days of age, indicating that 0 day-old chicks had a higher tolerance to CO_2 when the gas was inducing death.

Table 5. 2. The effect of immersion into 70, 80, 90 and 100 % CO_2 on the latency to behavioural indicators at 0, 3 and 6 days of age during gaseous euthanasia via immersion of broiler chickens.

Indicators*	Linear *P* value	Quadratic *P* value	Equation
Head shaking (a)			
0	0.19	0.51	
3	0.63	0.17	
6	0.20	0.39	
Gasping (a)			
0	0.45	0.73	
3	0.95	0.73	
6	0.67	0.14	
Loss of posture (i)			
0	<0.01	0.80	$y = -0.10x+21.18$
3	0.77	0.78	
6	0.93	0.99	
Cessation of rhythmic breathing (d)			
0	<0.01	<0.01	$y = 0.91x^2-171.89x+8069.71$
3	<0.01	<0.01	$y = 0.18x^2-32.74x+1533.19$
6	0.02	0.41	$y = -2.59x+297.94$
Cessation of movement (d)			
0	<0.01	<0.01	$y = 0.93x^2-173.58x+8134.55$
3	<0.01	<0.01	$y = 0.17x^2-31.37x+1482.01$
6	<0.01	0.59	$y = -2.92x+341.39$

* Behavioural indicator of: (a) distress, (i) insensibility, (d) death.

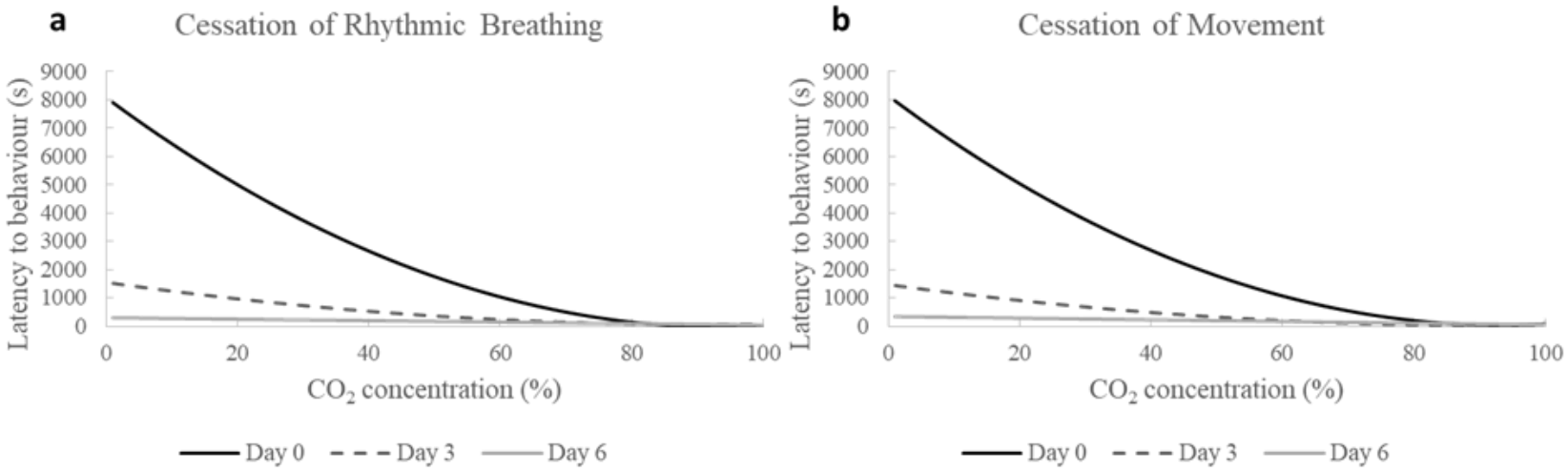

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Figure 5. 1. The effect of CO_2 concentration on the latency to cessation of rhythmic breathing (a) and cessation of movement (b) on young broilers at 0, 3 or 6 days of age in response to gaseous euthanasia via immersion induction.

Table 5. 3. Effect of immersion into four concentrations of CO_2 at three different ages on the latency to performance of behavioural indicators of distress, insensibility and death for young broilers.

Indicator *	n	CO_2 Treatment (%)					Age (days)					Interaction
		70	80	90	100	*P* value	0	3	6	*P* value	SEM	*P* value
Head shaking *(a)*	97	1.2	1.0	1.0	1.0	0.09	1.1	1.1	1.0	0.60	0.03	0.90
Gasping *(a)*	97	2.1	2.0	2.3	1.9	0.26	2.1^{ab}	1.9^{b}	2.2^{a}	0.05	0.07	0.99
Loss of posture *(i)*	97	15.3	13.8	15.7	13.8	0.23	12.4^{b}	13.9^{b}	17.8^{a}	<0.01	0.50	0.24
Rhythmic breathing *(d)*	97	251.1^{a}	98.4^{b}	38.8^{c}	37.5^{c}	<0.01	190.0^{a}	52.8^{b}	78.5^{b}	<0.01	15.05	<0.01
Cessation of movement *(d)*	97	260.8^{a}	112.2^{b}	49.5^{c}	49.2^{c}	<0.01	197.0^{a}	65.1^{c}	93.7^{b}	<0.01	14.77	<0.01

$^{a\text{-}c}$ Means within a row and main effect with different superscripts differ significantly at $P \leq 0.05$.

* Behavioural indicator of: (a) distress, (i) insensibility, (d) death.

Table 5. 4. Interaction between the age of birds and the CO_2 treatment on the latency to cessation of rhythmic breathing for young broiler chicks.

CO_2 treatment (%)	Age (day)		
	0	3	6
70	532.5^{a}	113.5^{b}	123.3^{b}
80	159.3^{b}	36.9^{d}	90.3^{bcd}
90	40.6^{cd}	33.2^{d}	42.5^{cd}
100	31.2^{d}	27.5^{d}	53.7^{cd}

[a-b] Means with different superscripts differ significantly at $P \leq 0.05$.

Table 5. 5. Interaction between the age of birds and the CO_2 treatment on the latency to cessation of movement for young broiler chicks.

CO_2 treatment (%)	Age (day)		
	0	3	6
70	536.5^{a}	123.2^{b}	138.0^{b}
80	163.3^{b}	48.0^{e}	120.0^{bcd}
90	48.6^{de}	48.1^{e}	51.9^{de}
100	43.9^{e}	40.9^{e}	62.9^{cde}

[a-b] Means with different superscripts differ significantly at $P \leq 0.05$.

5.4.1.2 Distress behaviours

An interaction effect of CO_2 treatment concentration and bird age occurred with the proportion of total time spent headshaking and gasping (Table 5.7 and 5.8). The lowest proportion of time spent performing these behaviours occurring with 70% CO_2 at Day 0, whilst the highest proportion occurred with 100% at Day 0 for headshaking, and 90% at Day 0 for gasping. This difference in the proportion of time is likely to be the result of latencies to death being the longest for Day 0 with 70%, and shortest for Day 0 at 90 and 100%. The duration of gasping was longest when birds were immersed into 70% CO_2, and the shortest when immersed into 80% or 100% CO_2 (Table 5.6). Frequency of gasping performance per second was higher with immersion into 80, 90 and 100% CO_2 compared to immersion into 70% CO_2. Duration and frequency of headshaking and duration of gasping were highest for 0 day old chicks. The frequency per second for gasping was longest for chicks at 6 days of age.

Table 5. 6. Effect of CO_2 induction treatment and age on the duration, frequency, frequency per second and proportion of total time spent performing behavioural indicators of distress during gaseous euthanasia of young broilers.

Indicator	CO_2 Treatment (%)					Age (days)					Interaction
	70	80	90	100	*P* value	0	3	6	*P* value	SEM	*P* value
Head shaking											
Duration (s)	5.78	5.52	4.77	5.64	0.33	7.09^{a}	4.42^{b}	4.78^{b}	<0.01	0.234	0.61
Frequency (n)	3.34	3.17	3.27	3.67	0.43	4.12^{a}	2.91^{b}	3.06^{b}	<0.01	0.131	0.22
Frequency per second	0.65	0.65	0.81	0.67	0.16	0.60	0.75	0.73	0.08	0.029	0.51
Proportion total time	0.04^{c}	0.07^{b}	0.10^{ab}	0.13^{a}	<0.01	0.10	0.08	0.07	0.12	0.005	<0.01
Gasping											
Duration (s)	7.88^{a}	5.43^{b}	6.29^{ab}	5.27^{b}	<0.01	7.37^{a}	6.28^{b}	5.08^{b}	<0.01	0.340	0.28
Frequency (n)	4.84	4.17	4.81	4.25	0.17	4.80	4.31	4.47	0.33	0.157	0.46
Frequency per second	0.69^{b}	0.82^{a}	0.83^{a}	0.84^{a}	0.01	0.67^{b}	0.78^{b}	0.93^{a}	<0.01	0.024	0.07
Proportion total time	0.05^{b}	0.08^{b}	0.14^{a}	0.12^{a}	<0.01	0.10^{ab}	0.11^{a}	0.08^{b}	0.03	0.007	<0.01

[a-c] Means within a row and main effect with different superscripts differ significantly at $P \leq 0.05$.
SEM = standard error of the mean

Table 5. 7. Interaction between the age of birds and the CO_2 treatment on the proportion of total time spent head shaking for young broiler chicks.

CO_2 treatment (%)	Age (day)		
	0	3	6
70	0.01^{e}	0.04^{de}	0.06^{de}
80	0.07^{cde}	0.09^{bcd}	0.06^{cde}
90	0.15^{ab}	0.08^{bcd}	0.08^{bcde}
100	0.16^{a}	0.13^{ab}	0.10^{abcd}

[a-e] Means with different superscripts differ significantly at $P \leq 0.05$.

Table 5. 8. Interaction between the age of birds and the CO_2 treatment on the proportion of total time spent gasping for young broiler chicks.

CO_2 treatment (%)	Age (day)		
	0	3	6
70	0.02^{e}	0.08^{bcde}	0.05^{cde}
80	0.06^{cde}	0.12^{abcd}	0.04^{de}
90	0.17^{a}	0.13^{abc}	0.12^{abcd}
100	0.14^{ab}	0.11^{abcd}	0.10^{abcd}

[a-e] Means with different superscripts differ significantly at $P \leq 0.05$.

5.4.1.3 Blood parameters

The age of chicks and the concentration of CO_2 that chicks were immersed into affected some blood parameters. CO_2 concentration and age acted interactively on the blood parameter range of pH less than 6.50 (Table 5.9). Day-old chicks immersed into 70% or 80% CO_2 had a higher percentage of pH values below 6.50 than all other combinations of age and immersion concentration except for 6 day-old chicks immersed into 70% CO_2 (Table 5.10).

The CO_2 concentration affected pH, with the percentage in each range differing between CO_2 treatments for pH ranges below 6.50, and between 6.70 and 6.89. The highest percentage of values with a pH below 6.50 occurred with 70%, then 80%, 90% and none in 100% CO_2 (Table 9). In the range of 6.70 to 6.89, the opposite response was found with the highest percentage of chicks with a blood pH in this range found for chicks immersed into 100%, then 90%, 80% and lowest for 70% CO_2. Chicks immersed in higher CO_2 concentrations had a higher percentage of blood pH values in the higher pH ranges, while immersion into 70% CO_2 had the highest percentage of bird in the lower pH ranges. This indicates that low pH occurs with 70% treatment and the higher pH occur with 100%. Blood pH was affected by concentration of immersion with

the majority of chicks immersed into 100% CO_2 having a blood pH in the 6.70 to 6.89 range, chicks immersed into 90 and 80% CO_2 had the majority of pH values in the 6.50 to 6.69 range, and chicks immersed into 70% CO_2 had pH values below 6.50 or between 6.50 and 6.69 (Table 5.9). Chicks immersed into 100% CO_2 had no pH values below 6.50. This suggests that the higher the concentration of immersion the less acidic the blood.

CO_2 concentration and age had no interactive effect on pCO_2 (Table 5.9). Although pCO_2 did not have a significant CO_2 concentration treatment effect, the 100% immersion treatment was the only treatment which resulted in chicks reaching pCO_2 of over 130 mmHg. An age effect was found for blood pCO_2 in the highest range (>130 mmHg). In this category, 100% of the chicks tested at both 0 and 6 days of age had pCO_2 levels higher than 130 mmHg, while only 61.2% those tested at 3 days of age fell in this category.

No interaction between CO_2 concentration and age was found for either pO_2 (Table 5.9). Although no significant effect of CO_2 concentration was seen, a similar numerical trend for pO_2 values was visible, with chicks immersed into 70% CO_2 having values in highest ranges, and chicks immersed into 90 or 100% CO_2 had lower pO_2.

Age affected the percentage of chicks found in three blood pO_2 range categories. Chicks at 0 days of age had the highest percentage of blood pO_2 values in the lowest range (between 25 and 50), whilst chicks at 3 days-old had the lowest percentage. In the higher pO_2 ranges, 3 day-old chicks had the highest percentage of pO_2 values in this range (51 to 75), or were the only age at which chicks had pO_2 values in this range (76 to 100). Overall, 0 day-old chicks had the most values with low pO_2 values, while 3 day-old chicks had the widest spread of pO_2 values and the highest pO_2 values.

Table 5. 9. Effect of immersion into different CO_2 concentrations at 0, 3 or 6 days of age on the percentage of birds (n=96) within a category for blood pH, pCO_2 and *pO2*.

% in category	CO_2 Treatment (%)					Age (day)					Interaction
	70	80	90	100	*P* value	0	3	6	*P* value	SEM	*P* value
pH											
<6.50	45.5^{a}	29.5^{ab}	9.1^{bc}	0^{c}	<0.01	43.3^{a}	3.1^{b}	16.7^{b}	<0.01	4.29	<0.01
6.50-6.69	45.5	54.5	61.4	39.1	0.45	43.3	56.2	50.0	0.55	5.24	0.12
6.70-6.89	4.5^{b}	15.9^{b}	29.5^{ab}	60.9^{a}	<0.01	13.3	37.5	33.3	0.06	4.72	0.84
≥6.90	4.5	0	0	0	0.48	0	3.1	0	0.42	1.12	0.52
pCO_2 (mmHg)											
>130	86.4	95.4	90.1	100.0	0.33	100.0^{a}	61.2^{b}	100.0^{a}	<0.01	2.67	0.28
130-101	4.5	4.5	4.5	0	0.83	0	9.4	0	0.08	1.92	0.94
100-71	9.1	0	4.5	0	0.34	0	9.4	0	0.06	1.92	0.3
≤70	0	0	0	0	-	0	0	0	-	0	-
pO_2 (mmHg)											
25-50	40.9	40.9	56.8	52.2	0.52	80.0^{a}	15.6^{c}	50.0^{b}	<0.01	5.23	0.75
51-75	45.4	40.9	34.1	43.5	0.86	20.0^{b}	56.2^{a}	46.3^{ab}	0.01	5.15	0.52
76-100	4.5	18.2	0	4.3	0.08	0^{b}	15.6^{a}	3.7^{ab}	0.03	2.67	0.51
101-125	4.5	0	9.1	0	0.34	0	9.4	0	0.06	1.92	0.32
>125	4.5	0	0	0	0.48	0	3.1	0	0.42	1.12	0.52

$^{a-c}$ Means within a row and main effect with different superscripts differ significantly at $P \leq 0.05$.

* pCO_2 = partial pressure of CO_2, pO_2 = partial pressure of O_2.

Table 5. 10. Interaction between the age of birds and CO_2 concentration into which chicks were immersed on the percentage of young broiler chicks with a blood pH below 6.50.

CO_2 treatment (%)	Age (day)		
	0	3	6
70	85.7[a]	12.5[b]	42.8[ab]
80	75.0[a]	0[b]	8.3[b]
90	12.5[b]	0[b]	16.7[b]
100	0[b]	0[b]	0[b]

[a-b] Means with different superscripts differ significantly at $P \leq 0.05$.

5.4.2 Experiment two

5.4.2.1 Latency to behavioural observations

Headshaking was the only behaviour that showed an interaction effect between CO_2 induction method and age, with headshaking occurring earliest for the immersion treatments regardless of day, and the latency being longer for the gradual induction at 3 days than at 6 days (Table 5.11). Induction method had a clear effect on behaviour, with the latency to performance of all behaviours monitored being shortest for immersion compared to the gradual flow rate treatment. A difference was also seen between the ages for gasping and cessation of rhythmic breathing, with these behaviours occurring earlier at 6 days of age than at 3 days of age.

Duration and proportion of gasping demonstrated an interactive effect of the CO_2 induction treatment and age (Table 5.12). Gasping duration was the longest for the gradual treatments at both ages when compared to the immersion treatments at both ages. The proportion of time spent performing gasping was the highest when gradual induction was used on day 6, followed by gradual induction on day 3, and the lowest with immersion. CO_2 induction method affected the duration and frequency of headshaking and gasping, with these behaviours being longer and more frequent with gradual treatment compared to immersion induction. The frequency of distress behaviour performance per second for gasping and headshaking, was higher with immersion induction then with gradual treatment. An age effect occurred for headshaking duration and frequency, with these being highest at 3 days, and for gasping duration, with this being longest at 6 days of age.

Table 5. 11. Effect of gradual or immersion induction treatments for gas euthanasia at 3 and 6 days of age on the time to performance of behavioural indicators of distress, insensibility and death.

Treatment Effects	Behavioural indicator* (s)				
	Head shaking*(a)*	Gasping*(a)*	Loss of posture*(i)*	Rhythmic breathing*(d)*	Cessation of movement*(d)*
CO_2 Induction Treatment[1]					
Gradual	10.9[a]	12.5[a]	56.9[a]	175.4[a]	178.1[a]
Immersion	1.1[b]	2.1[b]	13.9[b]	31.1[b]	43.4[b]
P value	<0.01	<0.01	<0.01	<0.01	<0.01
Age (day)[1]					
3	6.9[a]	8.1[a]	35.1	109.7[a]	115.8
6	5.1[b]	6.4[b]	35.7	96.8[b]	105.8
P value	<0.01	<0.01	0.91	0.03	0.26
Interaction (CO_2 induction treatment x Age)[1]					
3 Gradual	12.5[a]	14.0	56.2	187.3	189.2
3 Immersion	1.2[c]	2.3	14.0	32.1	42.4
6 Gradual	9.2[b]	11.0	57.6	163.4	167.0
6 Immersion	1.1[c]	1.9	13.8	30.2	44.5
P value	0.03	0.33	0.69	0.26	0.15
SEM	0.82	0.85	3.34	11.57	10.95

[a-c] Means within a column and main effect section with different superscripts differ significantly at $P \leq 0.05$.

[1] n= 44.

* Behavioural indicator of: (a) distress, (i) insensibility, (d) death.

Table 5. 12. Effect of CO_2 induction treatment and age on the duration, frequency, frequency per second and proportion of total time spent performing behavioural indicators of distress during gaseous euthanasia of young broilers.

Treatment Effects	Behavioural indicator							
	Headshaking				Gasping			
	Duration (s)	Frequency (n)	Frequency per second	Proportion	Duration (s)	Frequency (n)	Frequency per second	Proportion
CO_2 Induction Treatment								
Gradual	16.09[a]	5.95[a]	0.40[b]	0.09	34.00[a]	12.82[a]	0.38[b]	0.20[a]
Immersion	3.52[b]	3.02[b]	0.97[a]	0.08	4.23[b]	3.79[b]	0.93[a]	0.10[b]
P value	<0.01	<0.01	<0.01	0.50	<0.01	<0.01	<0.01	<0.01
Age (day)								
3	11.54[a]	4.95[a]	0.64	0.10	17.54	7.59[b]	0.60	0.13[b]
6	8.07[b]	4.02[b]	0.73	0.08	20.68	9.02[a]	0.71	0.17[a]
P value	<0.01	0.02	0.31	0.12	0.22	<0.01	0.06	<0.01
Interaction (CO_2 induction treatment x Age)								
3 Gradual	19.09	6.50	0.38	0.10	30.73[a]	11.64	0.38	0.16[b]
3 Immersion	4.00	3.41	0.91	0.10	4.36[b]	3.54	0.82	0.11[c]
6 Gradual	13.09	5.41	0.38	0.08	37.27[a]	14.00	0.35	0.23[a]
6 Immersion	3.04	2.64	1.03	0.07	4.09[b]	4.04	1.04	0.10[c]
P value	0.59	0.97	0.87	0.76	0.02	0.40	0.07	<0.01
SEM	1.186	0.317	0.059	0.006	2.375	0.732	0.053	0.010

[a-c] Means within a column and main effect section with different superscripts differ significantly at $P \leq 0.05$.

5.5 Discussion

Immersion into a chamber pre-filled to a concentration of 100% CO_2 is the most efficacious induction method for the euthanasia of neonates as it results in a rapid insensibility and death, with the shortest period prior to insensibility during which chicks could be conscious to the distress resulting from CO_2 exposure (Baker et al., 2019). Immersion of chicks on day of hatch into 100% CO_2 can induce insensibility and death significantly faster than a fast gradual induction, which displaces 28% of the chamber volume per minute and is at the upper limit of displacement rates recommended by the AVMA (2013) for gaseous euthanasia (Baker et al., 2019). Similar to the previously described results for chicks at 0 days of age from the study by Baker and colleagues (2019), results from these experiments indicate that chicks of 3 and 6 days of age euthanized via immersion into CO_2 concentration of 100% had shorter times to distress, insensibility and death than those euthanized via gradual induction. The duration, the frequency and the proportion of total time spent performing behaviours indicative of distress were also shorter when immersion was used as the gaseous euthanasia induction treatment rather than a gradual induction. This indicates that gradual induction does not become more efficacious then immersion induction as the bird ages within ages tested.

The concentration of CO_2 in to which chicks were immersed did not affect the latency to performance of behaviours indicating distress and insensibility, as concentrations used for the four immersion were much higher than the concentrations at which these behaviours occur. This is because behaviours indicative of distress, headshaking and gasping, occur at CO_2 concentrations between 0.4 and 1.2%, and the behaviour indicative of insensibility, loss of posture, occurs between 11-18% CO_2 in neonate chicks euthanized via gradual induction (Baker et al., 2019). The behaviours indicative of death, including the cessation of rhythmic breathing and the total cessation of movement, occur at CO_2 concentrations between 61 and 78% CO_2 when chicks are euthanized by gradual induction on day of hatch (Baker et al., 2019). These concentrations are reflected in the current results, with immersion into 100% and 90% resulting in the shortest latencies, 80% resulting in a medium latency and 70% having the longest latency to occurrence of these behaviours. Furthermore, immersion of day-old chicks into a concentration of 60% CO_2 did not result in death after 20 minutes of exposure. The long latency to death established with immersion into 70%, is longer than those previously found for chicks euthanized via gradual induction (Baker et al., 2019). Neonates euthanized by gradual CO_2

induction with a flowrate of 28% chamber volume added per minute, ceased both rhythmic breathing and movement within 4 minutes (Baker et al., 2019), whilst in the current study it took over 8 minutes for rhythmic breathing and total movement to cease for neonate chicks euthanized by immersion into 70% CO_2. Although the time to death was longer for immersion into 70%, the latency to distress and insensibility were still shorter with immersion into 70% than with gradual induction. For the immersion of neonates, a concentration of 80% CO_2 is the minimum required for the euthanasia method to result in insensibility and death faster than gradual induction, although the time to death is longer than 90 or 100% CO_2. When comparing immersion into a chamber pre-filled to a concentration of either 90 or 100% CO_2, the chicks showed similar times to insensibility and death, as well as the same latency to performance of distress behaviours and proportion of total time spent performing distress behaviours. This indicates that the efficacy of CO_2 induction via immersion into 90% is equivalent to induction via immersion into 100% CO_2.

Neonates have been shown to have an increased tolerance to carbon dioxide, with higher minimum CO_2 concentrations, longer latencies to death and exposure times required than adult birds (Raj et al., 1992; Raj and Whittington, 1995; Baker et al., 2019). This tolerance to CO_2 disappears with age as it is no longer present in adult birds, with the time to death decreasing as birds age. Furthermore, older birds are less resistant to lower concentrations of CO_2, with the impact of the concentration into which the birds are immersed on latency to death decreasing as birds age. Baker and colleagues (2019) suggested that the CO_2 tolerance found in neonates primarily affects the onset of death by increasing time to death and may influence onset of insensibility, but does not equate to an increased tolerance to distress. Our current research supports this with longer latencies to death being seen with 0 day old chicks, and the time to death decreasing with age. Furthermore, that the latencies to loss of posture were shortest with 0 day old chicks, and no effect was seen on headshaking, indicates that the neonate tolerance to CO_2 does not extend to insensibility and distress.

Exposure to CO_2, as occurs with CO_2 euthanasia, causes a rise in CO_2 in the blood and a drop in blood pH as hypercapnia and hypoxia occur. When chicks from 0 to 6 days post-hatch were euthanized via immersion induction with varying concentrations CO_2, the pCO_2 increased with all of the CO_2 concentration treatments. All the chicks immersed into 100% CO_2 and the

majority of those immersed into 70, 80 or 90% CO_2 had pCO_2 values in the highest range of more than 130 mmhg. Previous research investigating blood parameters in ducks and turkeys that were euthanized via CO_2 reported that the pCO_2 values rose to 187.1 mmhg and 224.0 mmHg (Gerritzen et al., 2006). Our method of analysing the pCO_2 had a ceiling reading of >130mmhg, thus it is uncertain whether pCO_2 measured in this work reached as high a level as those previously reported. Regardless, the rise in pCO_2 values seen with all the treatments indicates that the gaseous euthanasia via immersion results in an increase of CO_2 in the blood. The rise in CO_2 and pCO_2 was accompanied by a drop in pH, as all the chicks had a blood pH of 6.9 or below, which is lower than the normal pH value of 7.4-7.5 (Guo et al., 2008; Martin et al., 2010; Montesinos and Ardiaca, 2013; Reece, 2015b; Schaal et al., 2016). This confirms that all the concentrations used for immersion induction resulted in the desired acidification of the bloodstream, as is expected with hypercapnia and CO_2 euthanasia. Immersion into 100% CO_2 resulted in chicks having the highest amount of CO_2 in the blood; however, this was not reflected in the pH of the blood, with it having the overall highest pH values, and no pH values of below <6.50. Low pH occurred at a higher percentage with chicks immersed into 70% CO_2, suggesting that increased acidification was due to the extended exposure time to the CO_2. The longer time to cessation of rhythmic breathing means extra time for CO_2 to be inhaled and for the acidification of the blood.

The concentrations of bicarbonate ion ($HCO3^-$) were of interest in this study, as it was believed that it would provide insight into how the tolerance of neonates changes as birds age. *In ovo*, $HCO3^-$ is an important component of a compensatory mechanism in place to accommodate the high concentrations of CO_2 in the blood and air cell (Burggren et al., 2015); the tolerance of neonates to CO_2 has been suggested to be attributable to the high CO_2 environment of the egg and this compensatory mechanism (Jaksch, 1981; Baker et al., 2019). The I-stat analyser measures values for both pH and pCO_2 and uses these to calculate the value for $HCO3^-$ (Abbott Point of Care, 2017). The majority of the pCO_2 values in this experiment were beyond the maximum reference range for the analyser (>130). This means no $HCO3^-$ values were available for analysis to help elucidate the mechanism behind the increased CO_2 tolerance of neonates.

During gas euthanasia, insensibility and death occur as results of both hypoxia and hypercapnia, and hypoxia can be identified in the blood as a decrease in pO_2. Hypoxia was most

severe with chicks at 0 days of age, as these had the lowest pO_2 values. The 0 day old chicks with low pO_2 also had the longest latencies to death, indicating the large decrease in pO_2 value could be attributable to the additional time until death allowing for more time for the O_2 to be exhaled and depleted from within the body. Previous research reported for normal values pO_2 for broiler breeders, measured via the i-stat analyser, to be between 32-81 mmhg, with a mean pO_2 of 46 mmhg (Martin et al., 2010). Hybrid Warren chicks had mean pO_2 values, as measured by a blood sampling microsystem, of 30 mmhg and 70 mmhg for venous and arterial pO_2, respectively, on day of hatch. This increased to of 42 mmhg (venous) and 90 mmhg (arterial), and 35 mmhg (venous) and 90 mmhg (arterial) for three and six days post-hatch respectively (Tawaza et al., 1983). No normal values specific to broiler chicks are available in the literature, to my knowledge. When compared to the range of normal values for adult broilers or hybrid chicks, not all the pO_2 values in this experiment fell below this range. This may mean that the decrease in pO_2 as expected from the hypoxia occurring with CO_2 exposure may not have always occurred in our experiment. For immersion into 100% CO_2 the majority of the values fell below those in the normal range, and the majority of the values from birds exposed to either 90 or 80% CO_2 fell either within or below the range. With the immersion into a CO_2 concentration of 70%, there was a small percentage of chicks with values in the higher pO_2 ranges indicating a possible increase in O_2. This could be a result of the higher percentage of atmospheric O_2 available in the 70% treatment, however, retrospectively, the lack of baseline readings for broiler chicks measured by i-stat means there are no control values for comparison and it is not possible be certain of this.

5.6 Conclusion

The induction of gaseous euthanasia via the immersion of chicks into 100% CO_2 is the most efficacious at resulting in a rapid loss of consciousness and death, with the shortest duration and lowest performance of behaviours that suggest distress, regardless of age. However, achieving this may be difficult on a practical level. The immersion of chicks into a CO_2 concentration of 90% was similar in efficacy to immersion into 100% CO_2 in regards to time to onset of distress behaviours, insensibility and death. Although latencies to distress and insensibility for immersion into 80% are equivalent to those for immersion into 90% or 100% CO_2, the time to death is extended with immersion into 80% compared to the higher CO_2 concentrations. However, once the immersion concentration dropped to 70% CO_2, longer latency

to death, for both cessation of rhythmic breathing and cessation of movement occur, and were longer than those found with a gradual induction. Age affects the efficacy of immersion induction gaseous euthanasia with the time to onset of death decreasing as chicks age. Overall, gaseous euthanasia via immersion into 90 or 100% CO_2 is efficacious at inducing rapid insensibility and death, with a shorter exposure to distress from CO_2 than other induction methods.

5.7 Acknowledgements

The author would like to thank Steve Laycock for his help with the project, as well as Chicken Farmers of Saskatchewan, Poultry Industry Council, Canadian Poultry Research Council, Egg Farmers of Canada, Hybrid Turkeys, the Ontario Ministry of Agriculture, Food and Rural Affairs, and Natural Sciences and Engineering Research Council for funding this study.

6.0 Chapter Six - General discussion

6.1 Introduction

Throughout broiler production, euthanasia is an essential component of bird management and welfare. This project aimed to reduce distress of broilers requiring euthanasia by investigating welfare and efficacy of euthanasia methods that are used for neonates in a hatchery setting or on farm. Post-hatch and during broiler production, a portion of the birds will be diseased, ill, malformed, or for some reason unviable. If this results in suffering and there is no reasonable chance of recovery then ending the life of the bird may be the most humane option available, as prolonging a life of suffering, illness and discomfort compromises bird welfare (AVMA, 2013). The humaneness of euthanasia is dependent on many components involved in the process of euthanasia, and these are specific to the euthanasia methods; this discussion aims to examine in-depth the different factors to consider when making decision regarding the efficacy and welfare of broiler euthanasia methods.

6.2 Insensibility

Euthanasia requires a rapid and irreversible loss of consciousness, followed by brain death (AVMA, 2013; NFACC, 2016a). The reasoning behind a rapid loss of consciousness is that the faster insensibility occurs, the less time the bird is conscious to and possibly suffering from the euthanasia process. When insensible, the bird has lost awareness and is unable to perceive, interpret and integrate sensory information; thus are unable to experience states of suffering, pain or distress (Adam and Sheridan, 2008; Hemsworth et al., 2009; Von Holleben et al., 2010; Erasmus et al., 2010b; Benson et al., 2012a,b; AVMA, 2013; Verhoeven et al., 2015; Terlouw et al., 2016a). Insensibility results from dysfunction or destruction of the brain structures that hold consciousness, the brainstem, thalamus and pallium (Renier et al., 2005; Butler and Cotterill, 2006; Erasmus et al., 2010b; Martin, 2015; Terlouw et al., 2016a). Methods that result in direct damage to these brain structures are preferable for euthanasia, as they will result in instantaneous or almost instantaneous insensibility (NFACC, 2016a). Furthermore, a short latency to insensibility, specifically irreversible insensibility, are often more important than a short latency to brain death when assessing welfare and euthanasia (Jacobs et al., 2019).

Hypoxia is one method of euthanasia that leads to dysfunction of brainstem, thalamus and pallium (Erasmus et al., 2010b) and is utilized in a hatchery setting when gaseous euthanasia is

used. Gaseous euthanasia, such as with carbon dioxide (CO_2), does not result in immediate insensibility. The fastest time to insensibility for day-old broiler chicks with CO_2 euthanasia occurs with immersion induction into 100% CO_2, with insensibility occurring within 9 s (Baker, Chapter 5). Immersion into other concentrations of CO_2, including 70, 80 or 90% CO_2, resulted in a relatively rapid insensibility, occurring 13-15 s post introduction to CO_2 (Baker, Chapter 5). Gradual induction of CO_2, which involves slowly increasing the concentration of CO_2 in the chamber, has a much longer latency to insensibility; with the fastest gradual induction method taking upwards of 80% longer for insensibility to occur (Baker et al., 2019). The increased time to insensibility found with gradual treatments reflects negatively on the welfare of the birds, as there is an extended period in which the bird could be conscious to the adverse effects of CO_2 inhalation. This indicates that despite a gradual induction method being recommended by the majority of the literature (Coenen et al., 2000; AVMA, 2013), immersion induction as compared to gradual induction (faster time to insensibility) would be preferred for the euthanasia of neonates. The longer time to insensibility observed with gradual induction is likely due to the additional time taken for the inhaled gas to reach a high enough CO_2 concentration and a low enough oxygen (O_2) concentration for hypercapnic hypoxia to render the brain dysfunctional. Additionally, the time taken for the chamber to reach the necessary CO_2 concentration before insensibility occurs will also lengthen the time to insensibility. With an immersion, ranging from 70% -100%, the environment into which the chick is placed is immediately at a high enough CO_2 concentration to rapidly induce insensibility.

Cervical dislocation methods also induce insensibility via hypoxia. With cervical dislocation, the hypoxia occurs as a consequence of insufficient O_2 for metabolic demand as blood flow to the brain is reduced. As cerebral ischemia requires some time to develop, from the moment of carotid artery rupture to cellular dysfunction, there is a short period of time between the act of euthanasia and loss of sensibility. In comparison to other types of on-farm euthanasia methods, such as non-penetrative captive bolt devices (NPCD), these cervical dislocation methods have a longer latency to insensibility. Time to insensibility was under 2 s with Zephyr, were as it took an average of 28s for insensibility to occur with manual cervical dislocation (CD) and 49s with mechanical cervical dislocation with the Koechner Euthanasia Device (KED). With the Zephyr, the NPCD tested in our experiment, loss of posture occurred almost instantaneously (Baker, Chapter 2). Other NPCD also resulted in very short latency to insensibility, as the

nictitating membrane reflex, an indicator of insensibility was no longer present 2 seconds post euthanasia application with both the Turkey Euthanasia Device (TED) (Hulet et al., 2013) and the Cash Poultry Killer (Martin et al., 2018). The rapid onset of insensibility is attributable to the concussive force produced by the NCPD. The impact of the bolt delivers enough force to cause physical destruction of the neurons throughout the brain immediately rendering it unable to process sensory information and maintain consciousness (Erasmus et al., 2010c; Casey-Trott et al., 2014; Terlouw et al., 2016a). Concussion of the brainstem has been suggested to occur with CD (Gregory and Wotton, 1990; Sparrey et al., 2014; Martin et al., 2016) and in our study concussion appears to occur with CD when the dislocation occurs around S-C1 or C1-C2. Likely, the force used in the twist-stretch motion caused the cranium to compress and collide with the brainstem. When evaluating the welfare of on-farm methods with respect to their ability to induce rapid insensibility, the Zephyr is the fastest.

Cull birds are often dehydrated at the time of euthanasia; however, the data from this series of experiments demonstrates that water deprivation of up to 72 hours without access to water, did not influence the time to insensibility occurring with euthanasia (Baker, Chapter 3). Despite dehydration not increasing the time to insensibility, it is still in the best interest of the bird that euthanasia is not delayed and dehydration is avoided.

6.3 Death and success rate

Euthanasia methods are required to induce rapid insensibility and consistently result in a swift death. Death is often not immediate with all euthanasia methods, which is why a rapid insensibility is essential. However, death is the ultimate goal of euthanasia, meaning a euthanasia method must consistently result in death (AVMA, 2013; NFACC, 2016a). Reliability of a method and confidence that birds are rendered insensible followed by death will influence both the bird welfare and the producer's decision to implement its use on the farm. Unreliable euthanasia methods may require secondary euthanasia attempts or may mean there is a possibility of birds reviving once disposed/discarded of; both will compromise bird welfare.

Death is not immediate with CO_2 euthanasia for day-old cull broiler chicks regardless of the induction method used. Immersion induction, in which chicks are immersed into a chamber pre-filled to a CO_2 concentration to 90% or above, has the shortest time to death. When immersed into 90 or 100% CO_2 neonates ceased movement within 1 minute of CO_2 introduction,

whilst with immersion into 80% movement ceased within 2 minutes. When immersed into 70% CO_2 it took over 4 minutes for movement to cease (Baker, Chapter 5). Neonates euthanized via gradual induction, in which the CO_2 is gradually introduced into the chamber at a particular flow rate, took between 3 to 10 minutes to cease moving (Baker et al., 2019). This indicates that with neonates, gaseous euthanasia via immersion induction means death, as well as insensibility, occurs earlier than if euthanized via gradual induction. Notably, the latency to death for chicks immersed into 70% CO_2 was longer than for those euthanized via the fast flow rate of gradual induction. High concentrations of CO_2 (80% or above) are needed to achieve rapid euthanasia for newly hatched chicks (AVMA, 2013). The ability to consistently result in death is arguably more important than a short latency to death. Immersion induction, regardless of concentration, had a success rate of 100%, whilst gradual induction successfully resulted in death 91% of the time (Baker et al., 2019). Notably, when day-old chicks were immersed into a CO_2 concentration of 60%, a 20 minutes exposure time did not result in loss of rhythmic breathing nor movement, indicating that death had not occurred (Baker, Chapter 5). This suggests that gaseous euthanasia with immersion into concentrations of 80% CO_2 or above can consistently result in rapid death, while immersion into 70% CO_2 consistently resulted in death, but not a rapid death. Gradual induction with displacement rates within the AVMA (2013) recommended range of 10 to 30% of chamber volume added per minute was less reliable in regards to consistent death and took longer for death to occur.

Once birds are placed into production, euthanasia methods differ, and of these on-farm euthanasia methods, CD has both the shortest latency to death and is the most consistent at successfully inducing death when performed by a trained operator. When compared to CD, the time to onset of (brain) death is longer with both mechanical cervical dislocation devices and NPCD (Baker, Chapter 2). MCD have been found to result in a longer time to death when used for the euthanasia of multiple different poultry species. The time to death was longer with the KED, compared to CD, when used with broilers aged 7 to 50 days of age (Baker, Chapter 2; Chapter 3), with broilers at 36, 42 and 43 days of age (Jacobs et al., 2019) and for turkeys at one and three weeks of age (Woolcott et al., 2018b). With a novel MCD method investigated by Martin et al. (2018), it also took longer for death to occur than CD. In this series of experiments, CD also resulted in death faster than the Zephyr, and evidence from previous research investigating other NPCD, such as the Cash Poultry Killer found longer time before wing-

flapping, leg pedalling and cloacal movement ceased than with CD (Martin et al., 2018). With proper training, the reliability of CD is high, with CD having a 100% success rate for euthanasia of broilers, layers and turkeys (Erasmus et al., 2010a; Martin et al., 2016, 2018; Woolcott et al., 2018b). With captive bolt devices, including the Zephyr, incidences of misfiring and unsuccessful euthanasia attempts occur (Gregory and Wotton, 1990; Erasmus et al., 2010a; Martin et al., 2016; Woolcott et al., 2018a). In this series of experiments, the success rate for the Zephyr ranged from 88 to 100% (Baker, Chapter 2,Chapter 3), and previous research has found success rates between 83% and 93% when the Zephyr was used for the euthanasia of turkeys (Erasmus et al., 2010a; Woolcott et al., 2018a). Other captive bolt devices exhibited similar success rates, with the success rate for the TED being 98% (Erasmus et al., 2010a), and 72% for the modified Rabbit Zinger (Martin et al., 2016). A single study found a success rate of 100% for the TED when used with both turkeys and chickens (Hulet et al., 2013). The multiple reports of misfiring of the device or unsuccessful euthanasia attempt, as reported both within the scientific literature and as oral accounts from producers, means that the bird requires either a second application of the NPCD or a secondary euthanasia method. The misfiring or failing of a euthanasia attempt compromises bird welfare, as it prolongs time to death and suffering, and may cause additional distress. Furthermore it is unpleasant for the producer and the lack of reliability may hinder its implementation on farm. When assessing the welfare of on-farm euthanasia methods by the ability to reliably induce rapid death, then CD would appear to be the most reliable on-farm method.

Water deprivation extends the time taken for death to occur. Time to death increases as early as after 24 hours of water deprivation, and the time to death continues to increase as the length of water deprivation increases (Baker, Chapter 3). This suggests that when cull birds are dehydrated, birds should be euthanized as soon as possible. Whether dehydration has a deleterious effect on the success rate of euthanasia methods is unclear. There was no indication that dehydration affected the success rates of the two cervical dislocation methods, as both had a 100% success rate. The Zephyr, however, had a 95% overall success rate, with the unsuccessful attempts only occurring with water deprived birds (one bird deprived for 24 hours and one bird deprived for 48 hours) (Baker, Chapter 3). This may indicate that water deprivation effects the success of the Zephyr, but further research is needed. Notably, the unsuccessful attempts occurred at the later ages, namely with birds at 36 and 50 days old. Water deprivation and the

associated dehydration increases the time to death with on-farm euthanasia methods and it is unclear whether this affects the success rate of euthanasia and the euthanasia methods.

6.4 Suffering, pain and distress

Euthanasia ends pain and distress, by ending the suffering associated with disease, injury or illness. However, it should be recognised that the euthanasia process cannot always be performed with a complete absence of suffering and it is crucial to minimise the distress prior to insensibility occurring (AVMA, 2013). This includes recognising and minimizing the possible sources of pain or distress throughout the entire euthanasia process and associated with the euthanasia method used, such as (but are not limited to) distress during catching, handling, restraint, novel environments as well as the different components of the actual euthanasia process itself (Thornber et al., 2014; Martin et al., 2018). In order to limit suffering associated with euthanasia its vital to ensure a rapid onset of insensibility; limiting the time in which a bird could be conscious of suffering, and ensure that the possible ways suffering could occur with a specific euthanasia methods is understood, minimised and taken into account when making euthanasia decisions.

Inhalation of CO_2 is distressful and likely painful to birds (Lambooij et al., 1999; Hawkins et al., 2006; Raj et al., 2006; Turner et al., 2012; Gerritzen et al., 2013). In neonates, the concentration of CO_2 that chicks indicate is distressful is much lower than the concentration at which the chicks become insensible (Baker et al., 2019). Behavioural indicators of distress are also performed continuously until insensibility occurs, suggesting that this period before insensibility is distressful (Gerritizen et al., 2007; Baker et al., 2019). Neonates indicate distress with all the induction methods examined in this series of experiments, with distress behaviours performed until insensibility with both gradual and immersion induction (Baker et al., 2019). It is noteworthy that the latency to insensibility as well as the duration and frequency of distress behaviour performance are shorter or less when immersion induction into 80%, 90 or 100% CO_2 is used rather than gradual induction (Baker, Chapter 5; Baker et al., 2019). CO_2 euthanasia recommendations currently state a gradual flowrate is preferable as a mild and slow death without distress is preferable, however this is based on the assumption that a slower death means minimal pain and distress from CO_2 (Coenen et al., 2000; Hawkins et al., 2006; AVMA, 2013). The fact that distress behaviours occur regardless of induction method, and that the time to

insensibility and the duration and frequency of distress behaviours is reduced with immersion induction, suggests that immersion induction may be preferable when euthanizing neonates and broilers up to 6 days of age. Gaseous euthanasia is being investigated as a potential alternative to maceration, as the industry is moving away from using maceration. Although maceration was not part of this investigation, maceration may be a more humane euthanasia method for neonate chicks, as the method results in instantaneous insensibility and death, due to the immediate destruction of brain tissues, without the additional and extended distress observed with CO_2 inhalation and gaseous euthanasia (AVMA, 2013; AAAP, 2011).

The different on-farm euthanasia methods also present possible sources of suffering. For example, the KED had the longest latency to insensibility, meaning with KED use, there is a longer time in which the bird may be conscious and experience distress, pain or suffering. Furthermore, the evidence from radiography and gross pathology indicates that the KED causes dislocation by forcing apart vertebrae rather than the preferable twist and stretch motion seen with CD (Baker, Chapter 2). This may comprise bird welfare, as it resulted in a high number of severe and complex fractures of cervical vertebrae. These fractures have been previously reported to be unacceptable if they occur prior to insensibility as they could result in pain (AVMA, 2013; NFACC 2016a). To the best of the author's knowledge studies have not qualified or quantified pain associated with these particular fractures of the vertebral column, however, it has been established that other fractures, such as keel bone fractures, are experienced as painful by birds (Nasr et al., 2012). Despite this, the combination of the skeletal damage and an extended period of consciousness mean there is distress associated with the KED that needs to be acknowledged. Technical problems with devices, such as jamming and misfiring, which occur with the Zephyr, as well as other NCPD, like TED and Cash Poultry Killer (Martin et al., 2018; Woolcott et al., 2018a) are also a welfare concern. These technical problems cause distress as they delay the euthanasia process, extend the time the bird requires restraint and handling; furthermore, an unsuccessful or partial shot will exasperate suffering if the bird is not properly insensible or euthanized. Restraint can also be an issue when birds are suffering from leg or hip issues. When these birds are restrained by the legs or when CD is performed, during which force in exerted on the legs by pulling, these may cause additional distress and pain during the process (Martin, 2015). In these cases, a method via which legs are not used to restrain the bird or have force exerted on them during the euthanasia process may be a more welfare friendly option.

Distress, pain or suffering that occurs with euthanasia method greatly impacts on the welfare associated with the euthanasia process, and each of the euthanasia methods has potential sources of distress that need to be taken into account making decision surrounding the euthanasia of broilers whether in a hatchery or later in production.

6.5 Importance of operator ability

When assessing the welfare of euthanasia methods, the ability to result in rapid insensibility, consistently result in rapid death and minimise potential suffering associated with the euthanasia process all are key in decision making. However, the human element is also critical when assessing euthanasia and welfare. The safety of the operator and mental/emotional impact on the operator, the comfort level with the method and ability to perform the euthanasia, practicality, cost, ease of use, and the aesthetics of the method all also impact animal welfare (AVMA, 2013; NFACC, 2016a; Martin et al., 2018; Jacobs et al., 2019). All factors that may influence the ability of an operator to perform a euthanasia method are important considerations as for euthanasia to be humane, it must be performed well. The incorrect application of a euthanasia method will compromise bird welfare and result in additional suffering. Similarly, if personnel/operators are incapable or uncomfortable and thus unwilling to perform euthanasia via a particular euthanasia method and for that reason forgo euthanizing a bird that requires it, welfare is compromised. In this case, it may be favourable to utilize a method which is noted as comparatively having fewer welfare outcomes, rather than delaying the ending of the animal's life and extending the suffering and distress of the bird. Overall, all the euthanasia methods have drawbacks in regards to the human factor, that may impact their usage, and these should be considered when making decisions regarding euthanasia methods and welfare.

6.6 Limitations and future work

Alongside those previously mentioned, there were a few limitations to the research. One of the limitations was that complete blinding was not always possible for all parts of the experiment; however, steps were taken to reduce the effects of this. For the experiments investigating on-farm euthanasia methods, the same person conducted the macroscopic scoring of radiography and gross pathology and the ante mortem behaviour recording. The assigning of euthanasia methods at random and use of numeric identifiers for the macroscopic scoring samples reduced the possibility of recognising or associating the sample with a particular treatment. Furthermore, the histological analysis was blind to treatment. Another limitation with

a post mortem data collection was that only the extent of hemorrhage was scored; it may have been beneficial to record whether the carotid arteries were indeed ruptured. Similarly, the extent of skull fracture and hemorrhaging on the head was measured, but a documentation of the location where these occurred would have strengthened the study and allowed for an assessment of the relationship between Zephyr placement, extent of damage and success rate.

A final limitation to the research was that when investigating CO_2 euthanasia and age, data collection was performed on day 0, 3 and 6. If data was collected on a daily basis, this would have allowed for a better understanding on how the impact of CO_2 changes with age, both for the behavioural indicators of distress, insensibility and death and the blood parameters. Furthermore, as baseline values or reference ranges for pH, pCO_2 and pO_2 specific to neonates and chicks of 3-6 days of age are not available, and it would have been useful to take blood samples and obtain baseline values for these variables prior to gas introduction.

To strengthen the knowledge on the welfare of euthanasia methods, further work that clarifies the mechanism involved in the neonate CO_2 tolerance would be beneficial. Similarly, elucidating the effect of stocking density within a euthanasia chamber on the efficacy of CO_2 induction methods would be useful for the industry to understand the maximum number of chicks to be euthanized simultaneously within a chamber. Finally, further investigation into pain and other forms of suffering that occur with euthanasia methods, such as CO_2 or MCD, will help increase the scientific knowledge base from which decisions on euthanasia and bird welfare can be made.

6.7 Conclusions

Overall, when making decisions in regards to the efficacy and welfare of euthanasia methods, the key factors to consider are the time to insensibility, the reliability and success rate of a method, and the distress and suffering that is involved in the euthanasia procedure. The data outlined in this thesis demonstrates that none of the euthanasia methods investigated in this research performs perfectly in all these categories. This is important to keep in mind when making decisions in regards to euthanasia and bird welfare. It is also worth noting that all euthanasia methods require proper training and competence to ensure bird welfare. Although the welfare of the bird takes precedence when making decisions regarding euthanasia, it is essential that euthanasia is performed, and that it is performed correctly and timely. Incorrect euthanasia

or forgoing euthanasia will only further compromise the welfare of birds. Thus, the impact of a euthanasia method on an operator is important, as this will affect the success rate and euthanasia rate on-farm or in a hatchery. Ultimately, it is most imperative that birds that require culling are euthanized, and that this is done promptly and in a way that minimises pain and distress.

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